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Yoo-Mee
Leuven, 14 november 2013

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List of abbreviations

ADM	admission
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AMP	adenosine monophosphate
APACHE	acute physiology and chronic health assessment evaluation
AROC	area under the receiver operating characteristic curve
AST	aspartate aminotransferase
AU	arbitrary units
AUC	area under the curve
BA	bile acid
BMI	body mass index
BSEP	bile salt export pump
CA	cholic acid
CAR	constitutive androstane receptor
CBil	conjugated bilirubin
CDCA	chenodeoxycholic acid
CI	critical illness
CK	cytokeratin
CK7	cytokeratin 7
CLD	cholestatic liver dysfunction
CRP	C-reactive protein
CV	centrolobular vene
CYP	cytochrome P450
DCA	deoxycholic acid
DPD	2,5-dichlorophenyldiazonium
EPaNIC	Impact of early parenteral nutrition completing enteral nutrition in adult critically ill patients
FXR	farnesoid X receptor
GBS	gallbladder sludge
G-CA	glycocholic acid
G-CDCA	glycochenodeoxycholic acid

GGT	gamma-glutamyl transpeptidase
HDCA	hyodeoxycholic acid
HPRT	hypoxanthine phosphoribosyltransferase
ICU	intensive care unit
IFCC	international federation of clinical chemistry
IL	Interleukin
IQR	interquartile range
LCA	lithocholic acid
LOS	length of stay
MDR	multidrug resistance protein
MODS	multiple organ dysfunction syndrome
MRP	multidrug resistance-associated protein
NTCP	Na+/taurocholate co-transporting polypeptide
OATP	organic anion transporting polypeptide
OD	optical density
PBS	phosphate buffered saline
PN	parenteral nutrition
PT	portal tract
PXR	pregnane X receptor
RXRα	retinoid X receptor alpha
SAPS	simplified acute physiology score
SD	standard deviation
SEM	standard error of the mean
SHP	short heterodimeric partner
SOFA	sequential organ failure assessment
TBA	total bile acids
TBil	total bilirubin
T-CA	taurocholic acid
T-CDCA	taurochenodeoxycholic acid
TNFα	tumor necrosis factor alpha
ULN	upper limit of normality
US	ultrasonography
VDR	vitamin D receptor

Chapter 1: Introduction

1.1. Critical illness

Critical illness is the state in which the patient has to rely on intensive and advanced medical support to survive. Mechanical devices such as ventilators, dialysis machines and extra-corporeal membrane oxygenators, together with pharmacological agents are the core of his medical support. The cause of this critical illness may result from trauma, severe infection, major surgery and exacerbations of medical disease. This technological advance in the 1950 and 1960's allowed critically ill patients to survive beyond the acute phase of critical illness. Patients did not any more die of respiratory failure, uremic coma and hemodynamic collapse. Prolonged critical illness has been in fact the consequence of our medical improvements: a 'disease of medical progress' [1]. HIV/AIDS and cancer have also seen the evolution from acute death to chronic illness, on a different time scale though.

The distinction between acute and prolonged critical illness has been underpinned by the characterization of two distinctive inflammatory, metabolic and neuroendocrine paradigms [2-5]. In the acute phase of critical illness, patients are generally confronted with a strong inflammatory response, hypermetabolism, catabolism and activation of the anterior pituitary gland (such as TSH and GH) and resistance to its downstream hormones (such as T3 and IGF-I). When the patient is entering the prolonged phase of critical illness, the systemic inflammation is more moderate, going together with a continuing catabolism but this time with a decreased metabolic rate. The neuroendocrine system is now characterized by an exhaustion of the anterior pituitary gland and a restored sensitivity of the peripheral tissues to the downstream hormones of the anterior pituitary gland. Attempts to extrapolate endocrine treatment strategies, such as GH supplementation, from the acute to the prolonged phase of critical illness have failed. Likewise, extending the dearly needed aggressive interventions from the acute 'shock' phase into the prolonged phase have been disappointing across the board. Striving for supranormal oxygen delivery by aggressive hemodynamic support and red blood cell transfusions, aggressive mechanical ventilation and long antibiotic treatments have all negatively impacted patient outcome [6]. Some authors claim that the metabolic shutdown, and the ensuing organ dysfunction, in the prolonged phase of critical illness can be regarded as a hibernation-type response, which may be adaptive [5]. Therefore, prolonged critical illness is tightly linked to the Multiple Organ Dysfunction Syndrome (MODS). This new clinical syndrome emerged in critically ill patients in the early 1970's. After major emergency surgery, many patients experienced a progressive failure of multiple organs, often in a similar pattern: respiratory failure, hypotension, renal failure, jaundice and gastrointestinal bleeding. In a consensus conference in 1991 MODS was defined as 'the presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention' [7, 8].

There is nowadays a consensus among clinicians that acute and prolonged critical illness are different entities not only from a pathophysiological point of view but equally from the way it should be approached to treat the underlying pathophysiological mechanisms.

1.2. ICU jaundice: more than sepsis-induced cholestasis?

While jaundice and hepatic dysfunction have always been consistently present in MODS since the first descriptions in the early seventies, its definition, etiology, treatment have been lagging behind [9, 10]. Overt ICU jaundice, hallmarked by the yellow discoloration of the skin and the corneas and elevated serum bilirubin levels, has been typically associated with prolonged critical illness [11]. The presence of ICU jaundice has a strong association with increased ICU mortality [12]. Several contributing factors for ICU jaundice have been identified [13]. Sepsis is the main cause of this complication, certainly when it originates from intra-abdominal infections by Gram-negative bacteria. However, also pneumonia and endocarditis have been commonly linked to ICU jaundice. Moreover, the antibiotics to treat these infections during sepsis may contribute themselves to the ICU jaundice. For basically all antibiotics cholestatic liver dysfunction has been described: beta-lactam antibiotics, carbapenems, fluoroquinolones, glycopeptides, cephalosporines and antifungals. An increase of the bilirubin load through hemolysis certainly aggravates ICU jaundice. The most common causes of discrete hemolysis are polytransfusion, the transfusion of older red blood cells, resorption of hematomas, hypersplenism, and hemolysis in circulatory support machines such as the ECMO.

Hepatic ischemia and hepatocellular necrosis, during the early phase of critical illness herald the genesis of ICU jaundice in the prolonged phase of critical illness [11]. Hepatic ischemia in cardio-circulatory shock often leads to ischemic hepatitis. This ischemic hepatitis is characterized by a steep increase in serum AST and ALT concentrations (> 5-10x the upper limit of normality) [14]. While, this rarely results in hepatic failure and decreased hepatic synthesis capacity, it is almost invariably followed by rising bilirubin levels. Overt hepatocellular necrosis occurs less frequently in critically ill patients.

1.3. The role of parenteral nutrition

A final factor, often involved in ICU jaundice, is the prolonged administration of parenteral nutrition [15]. Although the precise etiology is still unknown, a combination of toxicity of parenteral nutrition, a lack of enteral nutrition and the presence of sepsis and frequent infectious episodes have been suggested. As hepatic immaturity also contributes to the development of parenteral nutrition associated cholestasis, it is inevitably more prevalent in neonates and infants [16]. The clinical presentation is steady rise in conjugated bilirubin levels and a mild increase in the transaminases in the course of prolonged parenteral nutrition administration, usually > 2 weeks. Caloric overload by dextrose, lipids as well as amino acids have been related to the development of parenteral nutrition associated cholestasis [17, 18].

Initially the parenteral nutrition associated cholestasis is essentially a functional cholestasis, decreased bile flow, which resolves quickly if the parenteral nutrition is discontinued. At histopathological level one can only detect intracellular and intracanalicular cholestasis. This may evolve to steatosis, periportal inflammation, bile duct proliferation, fibrosis and in the end biliary cirrhosis.

1.4. Liver histology of ICU jaundice

Cholestasis is in fact a dynamic phenomenon of impaired bile flow, which is obviously not reflected in static liver biopsies. Increased abnormally located presence of bile components in the liver represents cholestasis. Hence, the most prominent features of ICU jaundice are hepatocellular bilirubinostasis, Kupffer cell hyperplasia and portal infiltrates of monocytes. Canalicular and ductular bilirubinostasis can also be present. Proliferation of the bile ducts, called ductular reaction, is supposedly driven by the inflammation in the liver. The centrolobular region is preferentially affected, as it hosts the P450 microsomal enzymes, while it has the lowest pO_2, indicating that a metabolically active region vulnerable to energy failure due to ATP generation.

Rarely ICU jaundice is the result of extra-hepatocytic processes. These include mechanical obstruction of the bile ducts and inflammation-driven narrowing of the bile ducts. In cholangitis lenta the small bile ducts are affected, while the large ducts are the primary focus in progressive sclerosing cholangitis [9]. Even the gallbladder can be caught in the cholestatic processes during critical illness

[19]. Biliary sludge is the presence of sediment in the gallbladder. It is commonly found in critically ill patients after an ICU-stay of already a few days and is diagnosed by ultrasonography [20]. Decreased bile flow by gallbladder dysmobility and changes in the composition of the bile are the main factors. Major abdominal surgery, trauma, transplantation and the administration of parenteral nutrition and antibiotics such as ceftriaxone have been described as risk factors for biliary sludge. Gallbladder sludge not necessary results in gallstones. However, it can give rise to the serious complication of acalculous cholecystitis. Cholangitis and acute pancreatitis are infrequently the result of gallbladder sludge. Usually biliary sludge resolves when the patient is improving.

1.5. Molecular mechanisms of bile flow

Hepatobiliary transport systems are responsible for the bile formation [21]. Maintenance of cell polarity through the presence of tight junctions and an intact cytoskeleton are necessary to create distinctive basolateral and apical sides of the hepatocytes, as well as to seal off the bile canaliculi. Secondly, hepatobiliary transporters mediate the excretory function of the liver. Biliary components and lipophilic endo- and exotoxins are taken up from the blood in the sinusoids at the basolateral side of the hepatocyte. After uptake into the hepatocyte, these components are modified and then conjugated in processes such as methylation, sulphatation, acetylation, glucuronidation and glycine/taurine/glutathione conjugation. In general, modification and conjugation are meant to lower the toxicity of these toxins. Finally, they are transported over the canalicular membrane into the bile compartment. Hence, the normal vector of transport goes from the basolateral -blood- compartment to the apical -canalicular- compartment.

The export of these toxins carried out by the ATP-binding cassette (ABC) transporters at the basolateral and apical membrane of the hepatocyte. Hepatic uptake of bile acids at the basolateral membrane is mediated by the sodium-dependent bile salt transporter Na^+/taurocholate co-transporting polypeptide (NTCP) and several organic anion transporters (OATPs) [22, 23]. These transporters are not specific for bilirubin and bile acids only. NTCP actively co-transports bile acids and Na^+, using the Na^+ gradient, created by the Na^+/K^+ ATPase. The driving force for the OATPs is anion exchange, instead of the Na^+ gradient. Amphipathic organic anions, such as bile salts and their conjugates, hormones and xenobiotics use the OATPs, with OATP2 (OATP1B1) and OATP8 (OATP1B3) the most important members.

Unconjugated bilirubin is supposed to either passively diffuse through the basolateral membrane due to its highly hydrophobic character or to be transported by OATP2.
Inside the hepatocyte the bile acids, bilirubin and toxins are modified and conjugated and require an intact cytoskeleton to be intracellularly transported to the apical membrane. Transporting them across the apical membrane in the canaliculi happens against a steep concentration gradient. This requires a lot of energy as ATP use. The equivalent of basolateral NTCP is the bile salt export pump (BSEP) at the apical site. In contrast to the basolateral transporters, BSEP's substrate specificity is confined to the monovalent bile salts. As apical transport is the rate-limiting factor, BSEP basically drives the bile salt dependent bile flow.

The other transporters at the canalicular membrane are from the multidrug resistance protein or from the multidrug resistance associated protein family. Multidrug resistance protein 1 (MDR1) is only expressed in the apical membrane but also in the cholangiocytes, kidney and the intestines, to facilitate toxin elimination [24]. The canalicular multidrug resistance protein 3 (MDR 3) transporters translocate phosphatidylcholine from the inner to the outer leaflet of the apical membrane. Therefore it is often called the phosphatidylcholine flippase. Phosphatidylcholine and bile salts aggregate in the micelles of the bile to protect the biliary epithelium from the detergent properties of the bile acids. The multidrug resistance associated protein 2 (MRP2) transports divalent bile acids and a wide range of amphipathic conjugates, such as bilirubin diglucuronide and glutathione. MRP2 is also localized in the kidney and the intestines.

However, there are also efflux pumps located at the basolateral membrane. Multidrug resistance associated protein 3 (MRP3) and multidrug resistance associated protein 4 (MRP4) are the most important ones. They are normally expressed in hepatocytes at very low levels. During cholestasis and 'toxic stress' to the hepatocytes they are upregulated [25, 26]. When they are upregulated, bile acid transport is reversed back into the plasma [27]. Organic solute transporters (OSTalpha and OSTbeta) are similarly upregulated during cholestasis. Both MRP3/4 and OST transport bile acids and conjugated sterols, such as steroids and prostaglandins.

Hepatobiliary transport is heavily regulated at the transcriptional and post-transcriptional level. Nuclear receptors (NRs) and transcription factors that are predominantly expressed in the liver, such hepatocyte nuclear factors HNF1, HNF3 and HNF4 [28]. In physiological conditions farnesoid X receptor (FXR) is the 'bile acid sensor'. FXR activation is known to lead to repression of basolateral BA uptake (NTCP, OATP1B1) and BA synthesis. FXR activation at the same time induces canalicular (BSEP, MRP2, MDR3) and basolateral efflux systems (organic solute transporter alpha/beta) [29].

Recently it has become clear that the NRs vitamin D receptor (VDR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) have significant regulatory roles in BA metabolism and/or transport. PXR and CAR also function as master regulators of the defence against xenobiotic (e.g. drugs) and endobiotic toxicity. Most of the transporters are also under post-transcriptional regulation. Insertion and retrieval of transporter proteins from vesicles (vesicular targeting and redistribution), post-translational modifications such as phosphorylation and glycosylation are just a few examples. Hence, it is clear that measuring gene or even protein expression of the bile acid transporters will always remain a surrogate marker for their in vivo activity. In fact, the relationship between transporter activity and bile flow is also under the influence of many other factors.

1.6. Management of ICU jaundice and gallbladder sludge during critical illness

The cornerstone in the management of ICU jaundice and gallbladder sludge during critical illness is their prompt diagnosis [9, 30]. Serum liver function tests and ultrasonography are the essential diagnostic tools. Adequate management of the hemodynamic status and infections are the basic but aspecific steps in the treatment. Starting enteral nutrition, when possible, and intensive insulin therapy to maintain normoglycemia may help to prevent or resolve ICU jaundice and gallbladder sludge [20]. The administration of ursodeoxycholic acid, a highly hydrophilic bile acid, has been shown to improve biochemical cholestasis and clinical parameters in cholestatic syndromes such as primary biliary cirrhosis. However, its use in ICU jaundice is not supported by strong clinical evidence. The administration of corticosteroids can be considered in acute drug-induced or inflammation-induced cholestasis. But data are lacking for the use of steroids to tackle ICU jaundice.

1.7. Rationale for the study of the role of parenteral nutrition during critical illness

Sepsis and critical illness have been associated with the development of ICU jaundice and cholestasis. Similarly, prolonged administration of parenteral nutrition is linked to the cholestatic disorders. Although, the use of parenteral nutrition has been shown to aggravate the risk for ICU jaundice during critical illness in observational studies, causality has never been demonstrated. It is well known that it is much harder to reach the caloric goals with enteral nutrition when the patient is

more severely ill. Hence, ICU jaundice may just reflect severity of illness. The causal relationship between parenteral nutrition and ICU jaundice and cholestasis can only be proven in interventional studies. Therefore, a randomized controlled trial in human subjects and animal studies with differing parenteral nutrition schemes are needed to assess the impact of parenteral nutrition on the clinical outcome and on changes in bile acid transporter expression.

Changes in the biochemical profile, as well as in the molecular basis of cholestasis, need to be assessed in light of the difference between acute and prolonged critical illness. Overt conjugated hyperbilirubinemia in the prolonged phase of critical illness may not be the same as a mild increase of circulating bilirubin during the acute phase of critical illness. The cholestatic response may either be heralding nearing death, or a maladaptive response to critical illness, or a protective response in acute as well prolonged phase of critical illness.

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Chapter 2: Aim and study objectives

2.1. Aim

The aim of this thesis project is the assessment of cholestatic changes at biochemical and molecular level during critical illness. Their influence on outcome during critical illness will be evaluated in the context of a metabolic challenge, either caloric restriction or full nutritional support by parenteral nutrition.

2.2. General hypothesis

The central hypothesis of this doctoral research project states that 'cholestasis' in the early phase of critical illness is brought about by changes in bile acid synthesis and transport and is a protective response of the liver. Parenteral nutrition can modify this protective cholestatic response.

2.3. Specific study objectives

This hypothesis was tested by means of the following specific study objectives:

(1) To unravel the mechanisms behind the cholestasis during critical illness the expression of bile acid transporters and synthesis enzymes, together with the regulating network of nuclear receptors will be analyzed in liver biopsies of critically ill patients and control patients undergoing major abdominal surgery.

(2) The effect of caloric restriction or, on the other hand, nutritional support by parenteral nutrition on serum markers of cholestasis and the expression of bile acid transporters and synthesis enzymes, together with their regulating network of nuclear receptors will be studied in a validated rabbit model of critical illness.

(3) In a post-hoc analysis of a large randomized controlled trial in critically ill patients the effect of either caloric restriction or nutritional support by parenteral nutrition on the development of biochemical cholestasis and gallbladder sludge during the first week of critical illness will be examined.

Chapter 3:

Critical illness evokes elevated circulating bile acids related to altered hepatic transporter and nuclear receptor expression

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3.1. Abstract

Hyperbilirubinemia is common during critical illness and is associated with adverse outcome. Whether hyperbilirubinemia reflects ICU cholestasis is unclear. Therefore, the aim of this study was to analyze hyperbilirubinemia in conjunction with serum bile acids (BAs) and the key steps in BA synthesis, transport and regulation by nuclear receptors (NRs).

Serum BA and bilirubin levels were determined in 130 ICU and 20 control patients. In liver biopsies mRNA expression of BA synthesis enzymes, BA transporters and NRs was assessed. In a subset (40 ICU/10 controls) immunohistochemical staining of the transporters and receptors together with a histological evaluation of cholestasis was performed.
BA levels were much more elevated than bilirubin in ICU patients. Conjugated cholic acid (CA) and chenodeoxycholic acid (CDCA) were elevated, with an increased CA/CDCA ratio. Unconjugated BA did not differ between controls and patients.

Despite elevated serum BA levels, CYP7A1 protein, the rate-limiting enzyme in BA synthesis, was not lowered in ICU patients. Also, protein expression of the apical bile salt export pump (BSEP) was decreased, while multidrug resistance-associated protein (MRP) 3 was strongly increased at the basolateral side. This reversal of BA transport towards the sinusoidal blood compartment is in line with the increased serum conjugated BA levels. Immunostaining showed marked downregulation of nuclear farnesoid X receptor (FXR), retinoid x receptor alpha (RXRα), constitutive androstane receptor (CAR) and pregnane x receptor (PXR) nuclear protein levels.

Conclusion: Failure to inhibit BA synthesis, upregulate canalicular BA export and localize pivotal NR in the hepatocytic nuclei may indicate dysfunctional feedback regulation by increased BA levels. Alternatively, critical illness may result in maintained BA-synthesis (CYP7A1), reversal of normal BA transport (BSEP/MRP3) and inhibition of the BA sensor (FXR/RXRα) to increase serum BA levels.

3.2. Introduction

Almost 20% of the intensive care unit (ICU) patients develop ICU jaundice or cholestasis, which has been linked to an increased risk of mortality and length of stay [1, 2]. Currently there is no consensus on the definition of cholestasis during critical illness. Most commonly, routine laboratory measurements of bilirubin and alkaline phosphatase (ALP) / gamma-glutamyl transpeptidase (GGT) with different cut-offs are used [2-4]. Therefore, in clinical practice ICU cholestasis is the equivalent of conjugated hyperbilirubinemia. As a causal link between hyperbilirubinemia and worse outcome is missing, it may even be a biochemical epiphenomenon. Additionally, the reliability of hyperbilirubinemia as a marker of cholestasis in critically ill patients may be questionable, since there are many factors that can influence the levels of bilirubin. The weakness of bilirubin as marker of cholestasis during critical illness and the absent mechanistic underpinning of ICU cholestasis were the main driver for this study.

To date, the behavior and impact of bile acids (BAs) during ICU jaundice has been neglected, despite their crucial role in bile formation [5], lipid/cholesterol metabolism and energy and glucose homeostasis [6]. Also studies on the BA transporters and their regulatory network of nuclear receptors (NRs) has so far been focused on either chronic cholestatic liver disorders, such as primary biliary cirrhosis or familial intrahepatic cholestasis [7] or on acute animal models of sepsis [8]. Endotoxin-induced pro-inflammatory cytokines lead to reduced Na+/taurocholate co-transporting polypeptide (NTCP) and organic anion transporting polypeptide (OATP) expression [8]. Expression of the canalicular efflux pumps, bile salt export pump (BSEP) and multidrug resistance-associated protein (MRP) 2 is reduced during rat endotoxemia, while multidrug resistance protein (MDR) 1 expression is increased [8]. MRP3 and MRP4, inducible basolateral efflux pumps, are strongly upregulated and may serve as an alternative escape route for cytotoxic compounds from hepatocytes into sinusoidal blood [7].

BA metabolism and transporter function is regulated by a complex network of NRs, together with their co-activators and co-repressors [9]. In physiological conditions farnesoid X receptor (FXR) is the ‘bile acid sensor’. FXR activation is known to lead to repression of basolateral BA uptake (NTCP, OATP1B1) and BA-synthesis. FXR activation at the same time induces canalicular (BSEP, MRP2, MDR3) and basolateral efflux systems (organic solute transporter alpha/beta). Recently it has become clear that the NRs vitamin D receptor (VDR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) have significant regulatory roles in BA metabolism and/or transport [9].

The aim of this study was to examine a large cohort of critically ill patients to gain mechanistic insights into ICU jaundice, with a focus on BAs, hepatocytic transporters involved in bile production as well as their regulating NRs. An understanding of these mechanisms has the potential not only to expand our knowledge of hepatic metabolic dysfunction in the critically ill but it may also convey hints whether hyperbilirubinemia or increased serum BA are a biochemical epiphenomenon of a failing hepatobiliary system or a desired compensatory reaction during critical illness.

3.3. Material and methods

Patients and serum analysis

Post-mortem liver biopsies were taken from ICU patients (n=130), enrolled in two large randomized controlled trials studying the effects of intensive insulin therapy in critically ill patients. [10, 11]. All deaths occurred after a multidisciplinary decision to restrict therapy when further treatment was judged to be futile. All liver samples were harvested within minutes after death. For comparison, liver biopsies from 20 demographically matched patients (controls) undergoing an elective restorative rectal resection were obtained. All protocol and consent forms were approved by the Institutional Review Board of the Katholieke Universiteit Leuven. Written informed consent was obtained from all patients, or, when the patient was unable to give consent, from the closest family member. All liver biopsies were taken from liver segment IVb, snap-frozen in liquid nitrogen, and stored at -80°C until analysis.

For all ICU patients, blood samples were taken on admission and the last day of ICU stay. Liver biochemistry was analyzed by routine automated laboratory assays: total bilirubin, alanine aminotransferase (ALT); aspartate aminotransferase (AST), GGT and ALP. Blood samples from controls were taken pre-operatively. Sepsis was defined according to Bone criteria as suspected or documented infection on the day of admission to the ICU and fulfillment of at least two of the three criteria for the systemic inflammatory response syndrome (receiving ventilatory support, white-cell count ≤ 4000 or ≥12,000 per cubic millimeter, and body temperature ≤36°C or ≥38°C) [12]. Serum concentrations of cytokines were quantified by a multiplexed microbead suspension enzyme-linked immunosorbent assay (Biosource, Carlsbad, CA) using the Luminex 100 system (Luminex Corp, Austin, TX) as previously published [13].

Individual serum BAs were quantified by high performance liquid chromatography-mass spectrometry using authentic BA standards and deuterated internal standards [14].

Gene and protein expression on liver biopsies

Total RNA was isolated and quantified as previously described [15]. Commercial gene expression assays from Applied Biosystems were used and are listed in Supplemental table 3.1. Data are

expressed as fold increase relative to the mean of the control patients. Immunoblot analysis of CYP7A1 was performed as described in the online supplement.

Histological and immunohistochemical analysis

For histological and immunohistochemical analysis, liver sections from a randomly chosen subset of study patients (40 ICU and 10 controls) were used. Four µm-thick sections were cut from frozen samples and stained with hematoxylin and eosin for a general histological assessment. For evaluation of bilirubinostasis and ductular reaction, sections were stained by Hall's method and for cytokeratin 7 (CK7) (Dako, Glostrup, Denmark). For immunohistochemistry 5 µm-thick frozen sections were dried overnight at room temperature, fixed in acetone for 10 minutes and washed in PBS immediately prior to use. Sections were incubated with primary antibodies for 30 min at room temperature. The primary antibodies used are listed in Supplemental table 3.2. For the staining of CK7, OATP2/8, MRP3, MRP2, MDR1 and MDR3 the second and third step consisted of peroxidase-labelled rabbit anti-mouse and peroxidase-labelled swine anti-rabbit immunoglobulins (both Dako, Glostrup, Denmark). Secondary and tertiary antibodies were diluted (1:50 and 1:100, respectively) in PBS (pH 7.2) containing 10 % normal human serum. For the staining of BSEP, the slides were incubated with an anti-rabbit peroxidase conjugated Envision antibody (Dako) and subsequently incubated with a goat peroxidase anti-peroxidase complex (goat PAP complex; Dako). For NTCP staining a protein block was performed prior to the application of the primary antibody to counteract the strong sinusoidal staining and the secondary step consisted of peroxidase-labelled swine anti-rabbit IgG (dilution 1:100; Dako), followed by peroxidase-labelled rabbit anti-swine IgG (dilution 1:100; Dako).

For the staining of the NRs sections were incubated with the primary antibody for 30 minutes at room temperature and subsequently incubated for 30 min at room temperature with anti-mouse peroxidase-conjugated Envision antibody (Dako). All incubations were performed for 30 min at room temperature and followed by a wash in 3 changes of PBS for 5 min.

For all immunohistochemical stainings, 3-amino-9-ethylcarbazol (AEC) in 0.01% H_2O_2 was used as substrate-chromogen. The sections were counterstained with hematoxylin. Negative controls consisted of omission of the primary antibody and were consistently negative. To ensure uniform handling of samples, all sections were processed simultaneously. All immunohistochemical stained slides were evaluated for staining patterns and intensities by four observers (TR, YV, JW and LL). Histological changes i.e. portal inflammation; hepatocellular, canalicular and ductular bilirubinostasis;

ductular reaction; steatosis and centrolobular necrosis were graded using a semi-quantitative scoring system. BA transporter expression was semi-quantitatively graded as compared to what was deemed normal by the pathologist. For the assessment of NRs, intensity of nuclear localized staining was scored.

Statistical analysis

Statistical analysis was performed using Statview 5.0.1 (SAS Institute, Cary, NC). All quantitative values were assessed for normality. Values with normal distribution, and those that were normalized after logarithmic transformation, are represented as mean $\pm$ standard error of the mean (SEM) and were compared using the unpaired Student's t-test. The non-normally distributed data were represented as medians and interquartile range (IQR) (1^{st}-3^{rd}) and compared by the non-parametric Mann-Whitney U test. Nominal and ordinal variables (expressed as numbers and percentages) were compared by Fisher's exact test. Correlations between variables were calculated using either Pearson's or Spearman's rank correlation test. For all comparisons a *p*-value less than 0.05 was deemed significant.

Table 3.1 Baseline characteristics of control and ICU patients

	Control (n=20)	ICU (n=130)	*p-value*
Gender (% male)	65	64	*0.9*
Age (years)	68 ± 3	68 ± 1	*0.7*
BMI (kg/m^2)	25.1 ± 0,6	24.9 ± 0.4	*0.4*
LOS ICU (days)		10 (6-21)	
APACHE II (score)		19 (12-27)	
Diagnostic Group (n,%)			
Cardiovascular disease / high-risk cardiac or complicated vascular surgery		33 (25)	
Respiratory disease / complicated pulmonary or esophageal surgery		42 (32)	
Gastrointestinal or hepatic disease / complicated abdominal surgery		16 (12)	
Neurology / neurosurgery		14 (11)	
Hematology / oncology		9 (7)	
Solid organ transplant		1 (<1)	
Polytrauma		3 (2)	
Renal / metabolic		2 (2)	
Other		10 (8)	
Sepsis (n, %)		65 (50)	
Serum markers on admission			
CRP (mg/L)		131 (61-192)	
ALT (IU/L)		29 (15-59)	
AST (IU/L)		48 (27-95)	
Total Bilirubin (mg/dL)		1.18 (0.63-2.41)	
GGT (IU/L)		40 (25-78)	
ALP (IU/L)		175 (125-258)	
Serum markers on day of biopsy			
CRP (mg/L)	7 (4-28)	150 (88-219)	*<0.0001*
Cytokines			
TNFα (pg/mL)		5160 (8-23825)	
IL-1β (pg/mL)		6750 (18-19963)	
IL-6 (pg/mL)		92910 (535-269918)	
ALT (IU/L)	15 (14-18)	46 (26-125)	*0.0002*
AST (IU/L)	18 (15-21)	62 (36-157)	*<0.0001*
Total Bilirubin (mg/dL)	0.37 (0.29-0.46)	2.89 (1.08-8.87)	*<0.0001*
GGT (IU/L)	25 (20-46)	75 (41-204)	*0.004*
ALP (IU/L)	220 (180-242)	350 (209-689)	*0.02*
Total bile acids (μM)	0.62 (0.42-0.92)	6.88 (3.12-17.71)	*<0.0001*

Baseline characteristics for all control and ICU patients. Represented p-values are calculated for the comparison between ICU and control patients. All data are represented as mean ± SEM or median with IQR (25^{th}-75^{th} percentiles) as appropriate.

3.4. Results

Biochemistry in ICU and control patients

Baseline characteristics of ICU (n=130) and control (n=20) patients are described in table 3.1. The total ICU population, as well as the subset used for immunohistochemical analysis, was matched with control patients for gender, age and body mass index (Supplemental table 3.3).

Serum total bilirubin on the last day of ICU stay was 8-fold higher in ICU patients than in controls (Table 3.1) and the hyperbilirubinemia was predominantly conjugated. Compared to controls, serum ALP and GGT levels in ICU patients were 1.6- and 3-fold higher respectively (Table 3.1). In parallel, serum total BAs were 11-fold higher (*p<0.0001*) in ICU patients (Table 3.1), this increase being mainly attributable to conjugated BAs (Table 3.2). There was no effect of tight glycemic control on circulating bilirubin or BA levels. There was an increase in conjugation percentage for the primary BA cholic acid (CA) (98.3% in patients vs 55.6% in controls) and chenodeoxycholic acid (CDCA) (95.9% in patients vs 37.5% in controls) (*p<0.0001*). Serum levels of glycocholic acid (G-CA) were on average 83-fold increased in ICU versus control patients, while glycochenodeoxycholic acid (G-CDCA) was 34-fold higher in the critically ill population. Taurocholic acid (T-CA) was 22-fold and taurochenodeoxycholic acid (T-CDCA) was 39-fold increased in critical illness. Serum levels of the unconjugated BAs CA, CDCA and deoxycholic acid (DCA) did not differ between the two populations. The ratio of unconjugated CA/CDCA (0.5 in patients vs 0.3 in controls, *p=0.003*) as well glycoconjugated CA/CDCA (1.1 in patients vs 0.4 in controls, *p<0.0001*) was higher in critically ill patients. After logarithmic transformation, serum levels of total bilirubin correlated strongly with G-CA, G-CDCA, T-CA and T-CDCA on the day of biopsy as shown in figure 3.1. Changes in serum markers of cholestasis and bilirubinostasis in the subset of ICU patients used for immunohistochemical analysis were similar to those seen in the entire ICU population used for mRNA analysis (data not shown).

Serum levels for TNFα, IL-1β, IL-6 are shown in table 3.1. In over 80% of the ICU patients levels of IFNγ, IL-2, IL-4 and IL-5 were undetectable, while in the control patients all measured cytokines were below the assay detection limits.

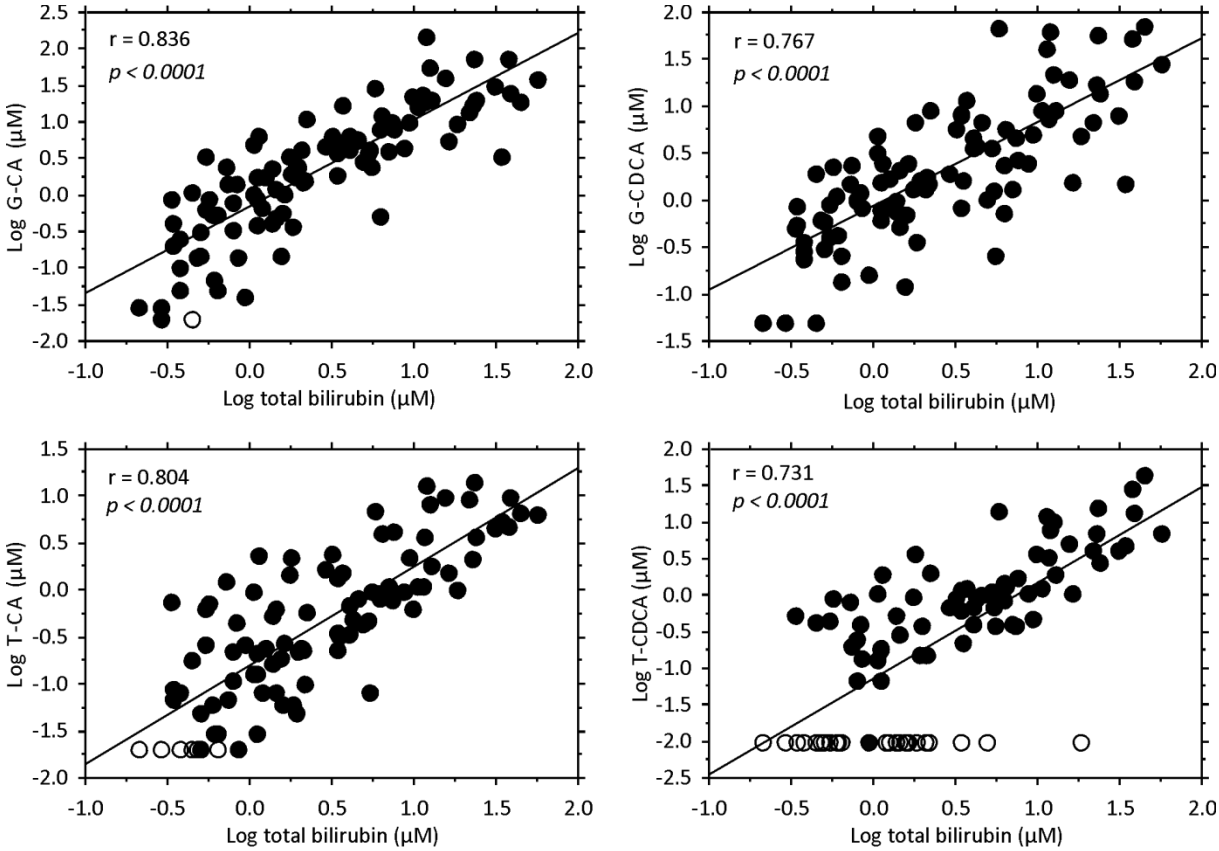

Figure 3.1 Correlation between serum levels of bile acids and total bilirubin

Correlation between serum levels of conjugated primary bile acids G-CA, G-CDCA, T-CA and T-CDCA and total bilirubin for ICU and control patients on day of biopsy. (○) Open dots represent values below detection limit.

Table 3.2 Serum levels of bile acids for control and ICU patients

Bile acid (µM)	Control (n=20)	ICU (n=130)	*p-value*
CA	0.04 (0.01 - 0.05)	0.05 (0.02 – 0.15)	*0.06*
CDCA	0.10 (0.09 – 0.15)	0.09 (0.05 – 0.18)	*0.5*
DCA	0.05 (0.05 – 0.08)	0.05 (0.05 – 0.13)	*0.4*
LCA*	0.04 (0.04 – 0.04)	0.04 (0.04 - 0.04)	*0.2*
UDCA*	0.01 (0.01 – 0.07)	0.01 (0.01 – 0.01)	*0.2*
G-CA	0.03 (0.03 – 0.09)	2.49 (0.68 – 7.25)	*<0.0001*
G-CDCA	0.05 (0.05 – 0.34)	1.70 (0.82 – 5.82)	*<0.0001*
G-DCA*	0.01 (0.01 – 0.01)	0.01 (0.01 – 0.14)	*0.05*
G-UDCA*	0.02 (0.02 – 0.02)	0.02 (0.02 – 0.13)	*0.1*
T-CA	0.02 (0.02 – 0.02)	0.43 (0.13 – 1.31)	*<0.0001*
T-CDCA	0.01 (0.01 – 0.01)	0.39 (0.01 – 1.16	*<0.0001*
T-DCA*	0.03 (0.03 – 0.03)	0.03 (0.03 – 0.03)	*0.1*
T-UDCA*	0.01 (0.01 – 0.01)	0.01 (0.01 – 0.01)	*0.7*
T-LCA*	0.01 (0.01 – 0.01)	0.01 (0.01 – 0.01)	*0.9*

Serum levels of bile acids for control and ICU patients on day of biopsy. Levels are expressed in µM and are represented as median with IQR (25^{th}-75^{th} percentiles). (*) more than 60% of the samples were below the detection limit.

Histological characteristics of cholestasis in ICU and control patients

Liver histology and immunohistochemical staining were performed in a random subset of 40 ICU patients and 10 controls (Table 3.3). The majority of the ICU biopsies exhibited typical histological features of intra-hepatic cholestasis (Figure 3.2). In 82% of the liver biopsies from the ICU patients hepatocellular and canalicular bilirubinostasis was present, while in 34% ductular bilirubinostasis also was present. This was absent in the control biopsies. A mild ductular reaction was seen in 20% of controls compared to ICU biopsies that showed a mild (37%) to severe (47%) ductular reaction. In 42% of ICU patients signs of cholangiolitis were observed. In contrast, the presence of portal inflammation did not differ between ICU patients and controls.

Morphological signs of cholestasis were linked with biochemical markers of cholestasis measured on the day of the biopsy. The degree of bilirubinostasis correlated with serum levels of total bilirubin ($\rho=0.816$, $p<0.0001$), ALP ($\rho=0.472$, $p=0.008$), GGT ($\rho=0.495$, $p=0.008$), G-CA ($\rho=0.775$, $p<0.0001$), G-CDCA ($\rho=0.726$, $p<0.0001$), T-CA ($\rho=0.739$, $p<0.0001$) and T-CDCA ($\rho=0.566$, $p=0.0001$). The presence of ductular reaction also correlated with the serum levels of total bilirubin ($\rho=0.709$, $p<0.0001$), ALP ($\rho=0.539$, $p=0.002$), GGT ($\rho=0.483$, $p=0.009$), G-CA ($\rho=0.591$, $p<0.0001$), G-CDCA ($\rho=0.598$, $p<0.0001$), T-CA ($\rho=0.696$, $p<0.0001$) and T-CDCA ($\rho=0.658$, $p<0.0001$).

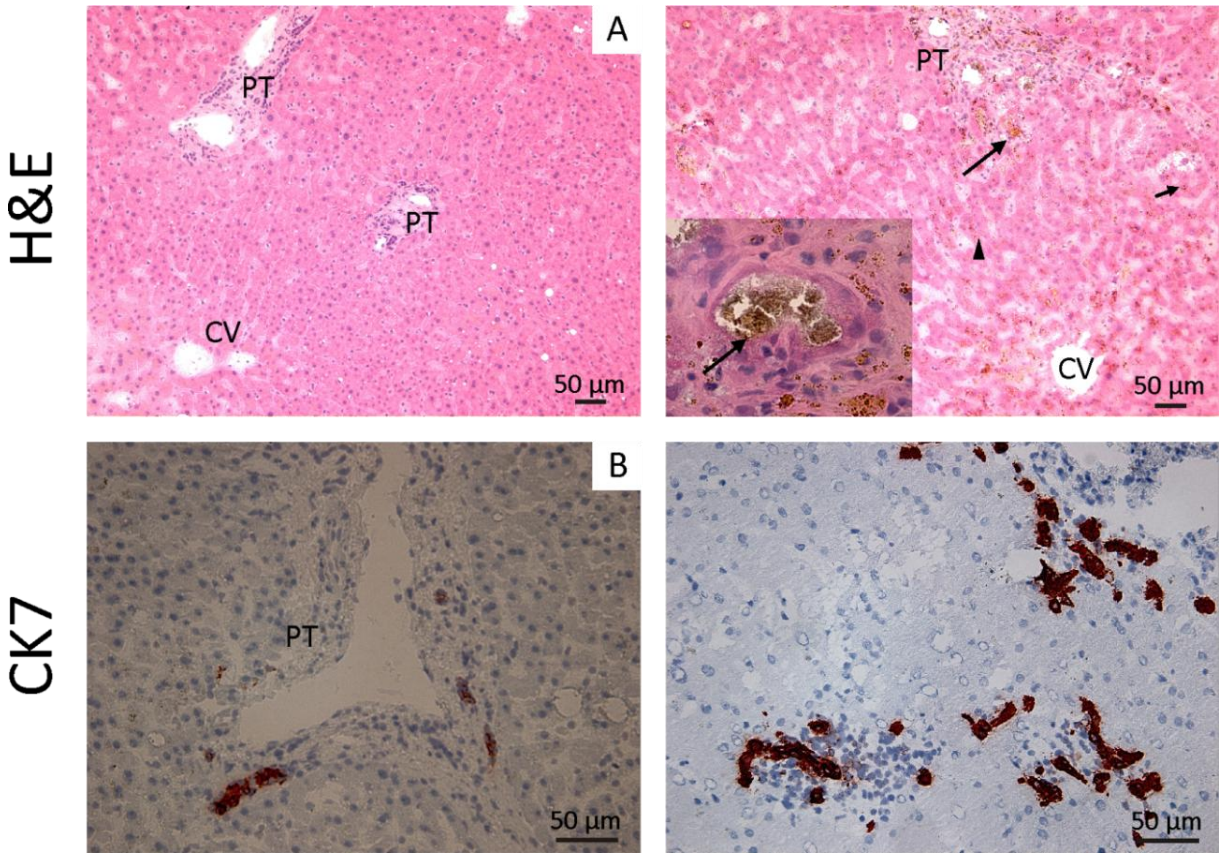

Figure 3.2 Representative liver sections for bilirubinostasis and ductular reaction

Left panel: Control patients. Right panel: ICU patients. A: Control patient with normal liver tissue with normal portal tract (PT) and no signs of cholestasis (left panel). Extensive cholestasis with hepatocellular (arrowhead), canalicular (short arrow) and ductular (long arrow) bilirubinostasis. PT with a dilated ductulus filled with a bile plug (right panel + small frame in the left lower corner) B: Normal CK7 staining of intralobular bile duct (left panel). Increased CK7 staining with ductular proliferation at the interface of the portal tract and the liver parenchyma (right panel).

Bile Acid synthetic enzymes in prolonged critically ill patients

Hepatic mRNA expression of CYP7A1, the rate-limiting step in BA synthesis was decreased by 94% in ICU patients compared to controls (*p<0.0001*), but CYP7A1 protein expression did not differ between the two groups. However, within the ICU group an inverse correlation between CYP7A1 protein and the serum levels of total BAs was observed. (*r=-0.347, p=0.0001*). In contrast, mRNA expression of CYP8B1, an enzyme involved in the synthesis of CA, was increased by 240% (*p<0.0001*).

Bile salt transporters in prolonged critically ill patients

In ICU patients, mRNA expression of the basolateral uptake transporters NTCP, OATP2 and OATP8 was downregulated compared with controls (Figure 3.3), but NTCP immunohistochemical staining did not differ between groups (Table 3.3). OATP2/8 staining had a clear intensity gradient from centrolobular to periportal regions in control patients. In 11/34 ICU patients a more uniform and extended staining with gradient fading was observed (Table 3.3).

Table 3.3 General histology, immunohistochemistry of hepatobiliary transporters and nuclear receptors of liver sections of control and ICU patients

	IHC score	Control (n=10)	ICU (n=40)	*p-value*
General	Bilirubinostasis			*<0.0001*
	0	10 (100)	7 (18)	
	1	0 (0)	17 (45)	
	2	0 (0)	14 (37)	
	Ductular reaction			*0.0009*
	0	8 (80)	6 (16)	
	1	2 (20)	14 (37)	
	2	0 (0)	11 (29)	
	3	0 (0)	7 (18)	
	Portal inflammation			*0.6*
	0	4 (40)	11 (29)	
	1	6 (60)	24 (63)	
	2	0 (0)	3 (8)	
Hepatobiliary transporters	NTCP			*0.7*
	-2	0 (0)	4(11)	
	-1	3 (33)	11 (29)	
	0	3 (33)	8 (22)	
	1	3 (33)	11 (30)	
	2	0 (0)	3 (8)	
	OATP2/8			*0.07*
	-1	0 (0)	5 (15)	
	0	7 (78)	11 (32)	
	1	2 (22)	11 (32)	
	2	0 (0)	7 (21)	
	MRP3			*<0.0001*
	0	6 (67)	3 (8)	
	1	3 (33)	9 (24)	
	2	0 (0)	26 (68)	
	BSEP			*0.02*
	-2	0 (0)	11 (28)	
	-1	2 (22)	16 (42)	
	0	7 (78)	11 (28)	
	MRP2			*0.02*
	0	8 (80)	13 (34)	
	1	2 (20)	10 (26)	
	2	0 (0)	15 (40)	
	MDR1			*0.05*
	0	9 (90)	18 (47)	
	1	1 (10)	13 (34)	
	2	0 (0)	7 (18)	
	MDR3			*0.0002*
	0	9 (90)	8 (21)	
	1	1 (10)	14 (37)	
	2	0 (0)	16 (42)	

Table 3.3 General histology, immunohistochemistry of hepatobiliary transporters and nuclear receptors of liver sections of control and ICU patients (Continued.)

		IHC score	Control (n=10)	ICU (n=40)	*p-value*
Nuclear receptors	CAR				*<0.0001*
		0	0 (0)	3 (8)	
		1	0 (0)	18 (47)	
		2	1 (11)	12 (32)	
		3	8 (89)	5 (13)	
	VDR				*0.2*
		0	1 (11)	13 (33)	
		1	6 (67)	13 (33)	
		2	2 (22)	14 (35)	
	FXR				*0.04*
		0	1 (10)	7 (18)	
		1	2 (20)	12 (31)	
		2	2 (20)	16 (41)	
		3	5 (50)	4 (10)	
	RXRα				*0.0004*
		0	1 (10)	3 (8)	
		1	0 (0)	14 (36)	
		2	0 (0)	14 (36)	
		3	9 (90)	8 (21)	
	PXR				*0.01*
		0	1 (10)	14 (35)	
		1	0 (0)	12 (30)	
		2	4 (40)	9 (23)	
		3	5 (50)	5 (13)	

Histological assessment (for bilirubinostasis, ductular reaction and portal inflammation), immunohistochemical assessment of the expression of hepatobiliary transporters and nuclear receptors of liver sections of 40 ICU and 10 control patients. Data are represented as numbers and percentages (between brackets). Represented p-values are calculated for the comparison ICU versus control patients.

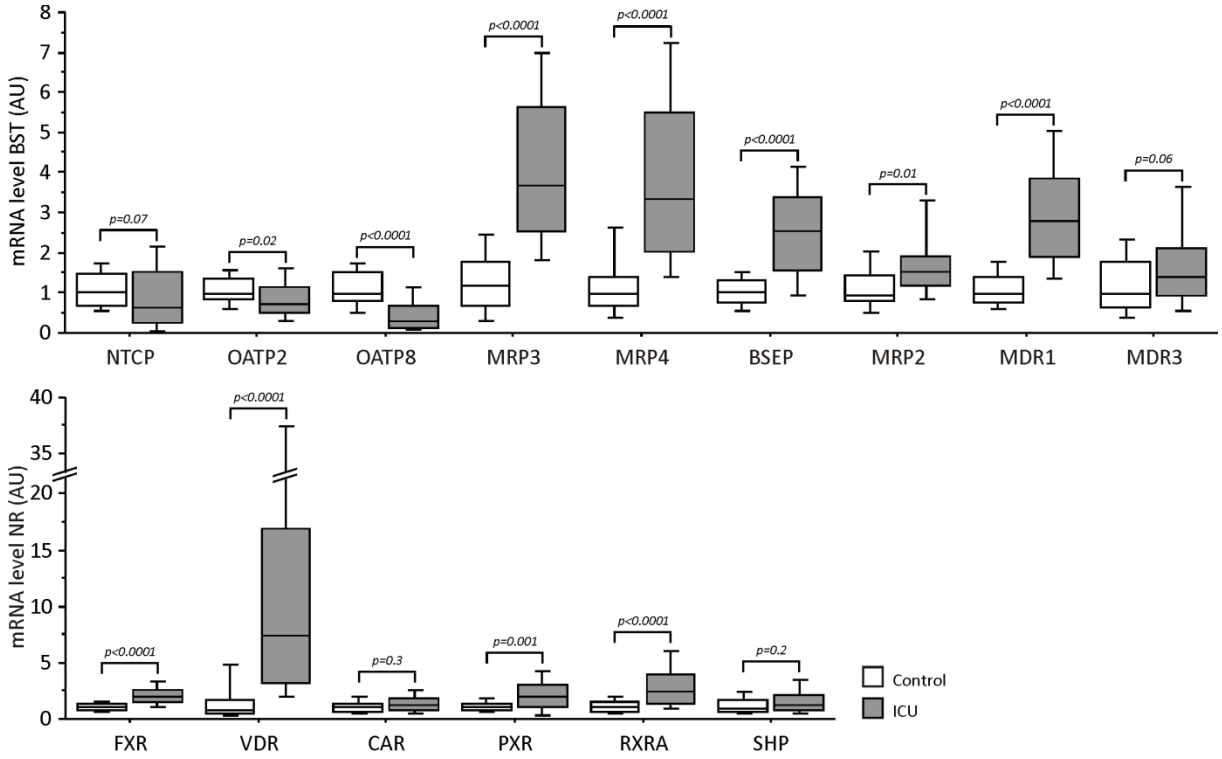

Figure 3.3 mRNA levels of hepatobiliary transporters and nuclear receptors in control and ICU patients

Upper panel: mRNA levels of hepatic basolateral influx pumps (NTCP, OATP2, OATP8), basolateral efflux transporters (MRP3, MRP4) and canalicular efflux pumps (BSEP, MRP2, MDR1, MDR3) of 130 ICU patients. Lower panel: mRNA levels of hepatic nuclear receptors (FXR, VDR, CAR, PXR, RXRA and SHP) of 130 ICU patients. mRNA levels are expressed relative to the mRNA expression of the housekeeping gene HPRT and relative to 20 control patients. Data are represented as median with IQR (25^{th}-75^{th} percentiles).

In contrast, mRNA levels of MRP3 and MRP4, the basolateral efflux transporters, were strongly upregulated. Immunohistochemistry confirmed a marked upregulation of MRP3 staining in ICU patients compared to control subjects (*p<0.0001*) (Table 3.3). Moreover, while controls only exhibited basolateral MRP3 staining in the centrolobular zone of the liver lobule, ICU patients showed a strong panlobular honeycomb staining pattern (Figure 3.4). For MRP3, mRNA and protein levels were in agreement (*ρ=0.432, p=0.004*). Moreover, MRP3 expression correlated positively with the degree of bilirubinostasis both at mRNA level (*ρ=0.529, p=0.0003*) and at protein level (*ρ=0.591, p<0.0001*). There was also a strong correlation between the MRP3 protein levels and biochemical markers of cholestatic liver dysfunction i.e. the serum levels of total bilirubin (*ρ=0.625, p=0.0003*), GGT (*ρ=0.519, p=0.005*), ALP (*ρ=0.551, p=0.002*), G-CA (*ρ=0.494, p=0.0008*) and G-CDCA (*ρ=0.484, p=0.001*). Due to technical limitations, we were not able to stain for MRP4.

mRNA expression of the canalicular efflux pumps BSEP, MRP2, MDR1 and MDR3 was significantly higher in ICU patients compared with control patients (Figure 3.3). In contrast to the increased mRNA

expression, protein expression of BSEP was downregulated (Table 3.3). The normal regular BSEP immunohistochemical staining pattern became irregular and discontinuity was observed in cholestatic zones. Severely cholestatic areas had no discernable immunostaining. In concert with the mRNA expression, MRP2 immunostaining was upregulated in ICU patients in comparison with controls (*p=0.02*) and correlated well with the degree of bilirubinostasis (*ρ=0.512, p=0.0004*), ductular reaction (ρ=*0.433, p=0.003*) and the serum levels of total bilirubin (*ρ=0.502, p=0.003*). MDR1 and MDR3 staining was also upregulated. At the canalicular domain of the hepatocytes, a fine linear MDR3 pattern, seen in control subjects, evolved towards a strong double strand pattern of staining around multiple dilated canaliculi in ICU patients (Figure 3.4). This is indicative of a very strong upregulation of MDR3 protein. Similar to MRP3, MDR3 protein levels correlated with the degree of bilirubinostasis seen on the liver sections (*ρ=0.569, p<0.0001*) and serum levels of total bilirubin (*ρ=0.745, p<0.0001*), GGT (*ρ=0.402, p=0.03*), ALP (*ρ=0.437, p=0.01*), G-CA (*ρ=0.639, p<0.0001*) and G-CDCA (*ρ=0.548, p=0.0002*) MDR1 protein staining showed a similar upregulation as MDR3 staining (*p=0.05*).

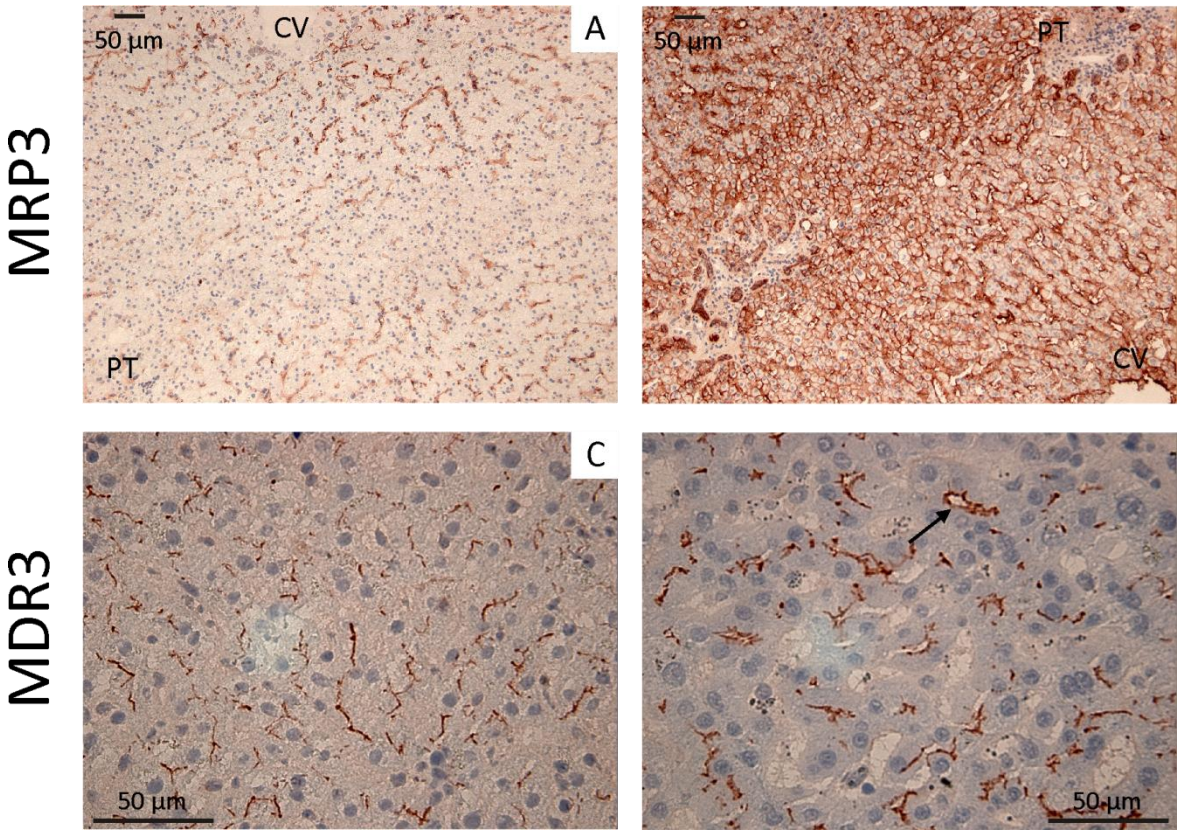

Figure 3.4 Representative liver sections for MRP3, MDR3

Left panel: Control patients. Right panel: ICU patients. A: Normal basolateral MRP3 staining showing clear centrolobular and midzonal activity (left panel). Markedly upregulated panlobular honeycomb MRP3 staining pattern. (right panel) B: Normal pattern of a fine canalicular linear MDR3 staining (left panel). Strong double

stranded pattern of MDR3 staining around multiple dilated canaliculi (right panel). (Definitions of abbreviations: MRP Multidrug resistance-associated protein, MDR multidrug resistance protein, CV Centrolobular vene, PT Portal tract)

Nuclear receptors in prolonged critically ill patients

In ICU patients, mRNA expression of the NRs FXR, VDR, PXR and RXRα was upregulated in comparison with control subjects. mRNA expression of CAR and SHP did not differ between groups. (Figure 3.3). In contrast to the increased mRNA expression, FXR, PXR and RXRα immunostaining in the nuclei was effectively absent in ICU patients while being clearly visible in controls (Table 3.3 and Figure 3.5). VDR protein expression did not differ between ICU and control patients.

Nuclear CAR staining was clearly decreased in ICU patients. Control subjects showed both cytoplasmic and intense nuclear staining, with a clear intensity gradient from periportal to centrolobular regions, whereas ICU patients only showed discrete positive cytoplasmic staining and a marked reduction in nuclear staining (Figure 3.5).

Overall there was no correlation between mRNA and protein levels for all NRs. In contrast, nuclear staining correlated inversely with histological and biochemical cholestatic parameters. For example, patients with lowest levels of nuclear CAR and RXRα staining, demonstrated the most severe bilirubinostasis. Serum levels of total bilirubin on the day of biopsy inversely correlated with the nuclear immunolocalization of CAR ($\rho=-0.589$, $p<0.0006$), FXR ($\rho=-0.416$, $p<0.01$) and RXRα ($\rho=-0.553$, $p<0.001$). RXRα staining also correlated well with BSEP apical protein visualization ($\rho=0.581$, $p<0.0001$).

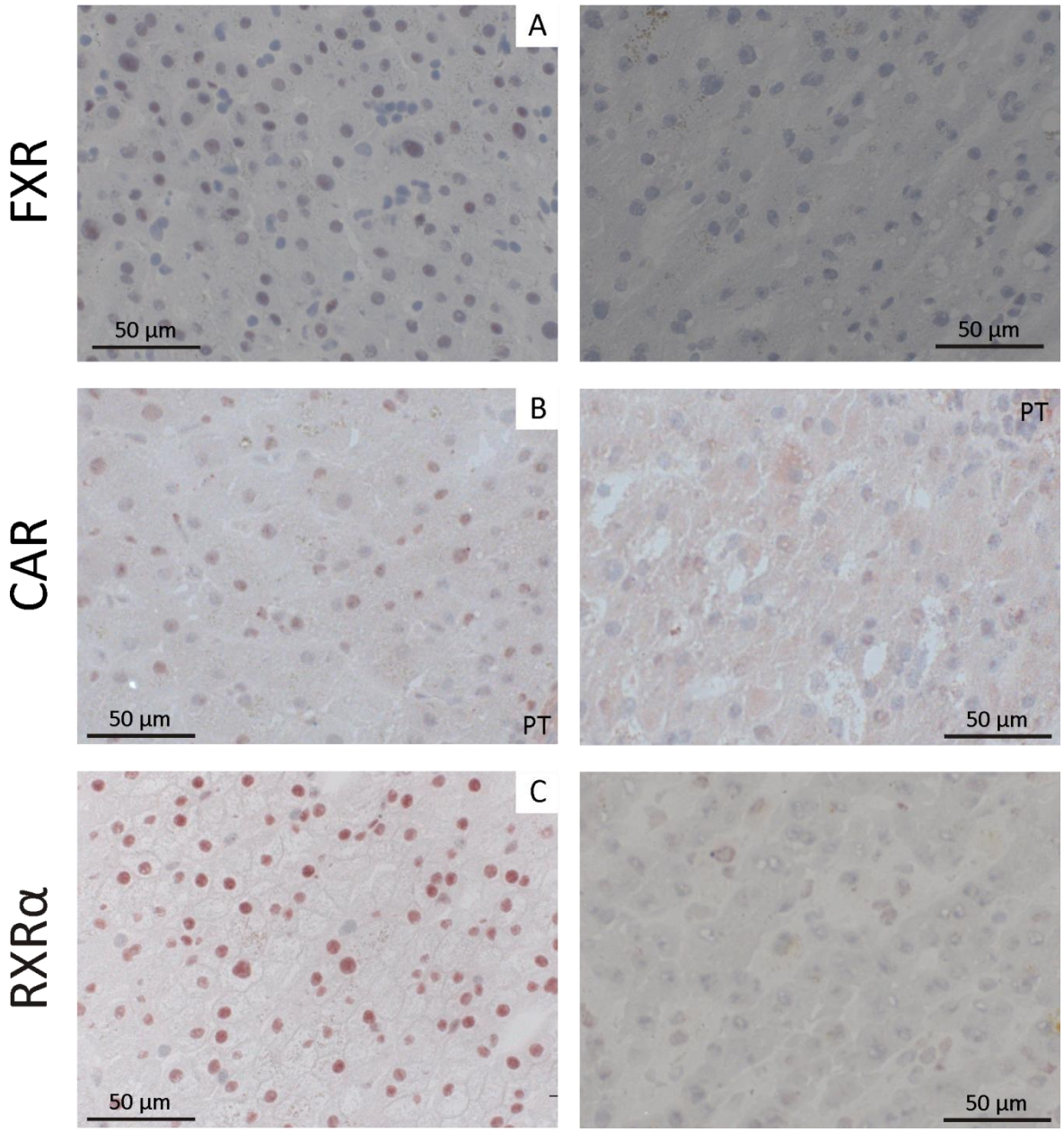

Figure 3.5 Representative liver sections for FXR, CAR and RXRα

Left panel: Control patients. Right panel: ICU patients. Normal liver with nuclear FXR (A), CAR (B) and RXRα (C) immunostaining clearly present in controls (left panel). Strongly decreased FXR (A), CAR (B) and RXRα (C) nuclear immunostaining in ICU patients (right panel).

3.5. Discussion

This study of post-mortem liver biopsies in conjunction with pre-agonal serum analyses found that BA levels are much more increased during critical illness than the bilirubin concentrations. Critical illness was also associated with maintained CYP7A1 levels, decreased apical BSEP protein, increased basolateral MRP3 protein expression. Nuclear localization of FXR and its heterodimeric partner RXRα was diminished in critically ill patients.

While bilirubin levels increased 8-fold during critical illness, the larger increase in circulating total BAs mainly consisted of glycine and taurine conjugates of CA and CDCA. Unconjugated CA and CDCA did not differ from controls. This indicates that the hepatocytes are able to conjugate potentially toxic BAs, either de novo synthesized or entero-hepatically recirculated. It also suggests that the transport of the conjugated BA towards the apical bile canaliculi is strongly shifted to the blood. The ratio of CA to CDCA was also increased in critically ill patients, consistent with the increased expression of hepatic CYP8B1 mRNA. This shift may represent a reduction in FXR-mediated FGF19 production by small bowel enterocytes due to reduced BAs being excreted into the gut, as FGF19 has recently been recognized to repress CYP8B1 in mice [16].

Despite the strongly elevated serum BA levels during critical illness, CYP7A1, the rate limiting step in de novo BAs synthesis was only repressed at the mRNA level but not at the protein level. This is in line with the absence of increased SHP mRNA expression in ICU patients, which mediates BA repression of CYP7A1 [17]. Furthermore, FXR and its heterodimeric partner RXRα, which act in concert with SHP to suppress BA-synthesis enzymes, were absent from the hepatocytic nucleus, where they exert transcriptional activity through direct binding to DNA. This may imply an at least partial loss of the sensing of BAs and its feedback regulation of de novo BA production, in light of the increased circulating BAs in ICU patients. Alternatively, critical illness may induce elevated BA levels by suppressing the BA sensor FXR and maintaining (CYP7A1) and/or shifting (CYP8B1) BA-synthesis. Cytoplasmic retention of RXR has also been found in models of acute liver inflammation [18, 19] and advanced extrahepatic cancer [20]. In the present study other NRs relevant to BA-regulation, namely PXR and CAR, also did not localize to the nucleus. The lower nuclear levels of PXR and CAR may not only affect bile formation, but also metabolic processes in the liver, such as energy homeostasis [21].

BAs, and bilirubin, are transported by the hepatocyte via the hepatobiliary transporters. In this study, the most prominent changes in the expression profile of the hepatic BA transporters during prolonged critical illness were observed in the basolateral efflux transporters MRP3 and MRP4.

Normally, MRP3 and MRP4 are expressed at very low levels in hepatocytes, but they become upregulated by inflammation and during longstanding cholestasis, presumably shifting transport of BAs back into sinusoidal blood for elimination by the kidneys [7]. Immunohistochemical expression of BSEP in the hepatocyte canalicular domain was dramatically reduced in ICU patients, especially in regions of bilirubinostasis, despite an increase in BSEP mRNA expression. Decreased expression of BSEP is a major contributor [22] to the cholestatic phenotype of the prolonged critically ill patient, as BAs will accumulate within the hepatocytes. In contrast to findings from chronic cholestatic disorders [7] and animal models of cholestasis [23] and sepsis [24], MRP2, the main canalicular bilirubin transporter, was upregulated during critical illness. This seems difficult to reconcile with the elevated serum bilirubin levels. Nevertheless, it may fit with the rather moderate increase in serum bilirubin, compared to the changes in serum BA concentrations. Besides, bile formation is a secretory process that depends on osmotically active solutes, mainly BAs. If the bile flow is hampered as a consequence of retained BAs, bilirubin will also be retained, essentially as a biochemical epiphenomenon.

The data on changes of BA synthesis and disposition and their regulation by the NR, do not allow to state whether they are beneficial or a failing compensatory response. Data from hepatocyte-RXRα-null mice indicate that these mice are protected against WY-14,643-induced liver injury by the upregulation of Mrp3 expression and increased efflux of BAs into blood for renal excretion [25]. FXR knock-out mice have a lower mortality rate and less liver injury during bile duct ligation. These FXR knock-out mice strongly increased Mrp4 and reduced Bsep expression [26]. However, FXR knock-out mice exhibit more hepatotoxicity when challenged with a cholic acid enriched diet [27]. Also, FXR agonists could be beneficial for patients with cholestatic liver diseases [28]. CAR knock-out mice show lower levels of serum and liver primary BAs than wild type mice during bile duct ligation [29]. Moreover, these CAR knock-out mice are resistant to acetaminophen liver toxicity [30]. Similarly, an increased bilirubin clearance has been demonstrated in PXR knock-out mice [31].

Histopathology of ICU patient liver biopsies revealed classic changes of cholestasis, namely bilirubinostasis, ductular proliferation and variable inflammation. Increased levels of serum bilirubin and conjugated BAs correlated strongly with the microscopic signs of bilirubinostasis and ductular reaction. Ductular proliferation and ductular differentiation of the hepatocytes are considered part of an adaptive, protective response to cholestasis. Canalicular MDR3 was also upregulated in ICU patients. Given the key role of biliary phospholipids in protecting bile duct epithelium from the potentially toxic biliary content, upregulation of MDR3 might also exert a compensatory action, protecting the canalicular membrane and biliary epithelium. Since MRP3 correlated well with histological bilirubinostasis and serum bilirubin and conjugated BAs levels, MRP3 upregulation is a

likely compensatory reaction to cholestasis, as has been observed in animal bile duct ligation models of cholestasis [32]. The upregulation of MRP3 (and MRP4) provides a mechanism to limit hepatocellular retention of hydrophobic BAs and other potentially toxic compounds that would normally be destined for biliary excretion. This is in keeping with the selective increase in serum taurine and glycine conjugated BAs, which have been conjugated by hepatocytes and transported back into the circulation. MRP3 upregulation has also been shown in acute sepsis models without longer lasting cholestasis [33]. Unexpectedly, there was a lack of association between ICU cholestasis and markers of inflammation, suggesting that inflammation is not the main contributor to cholestasis in prolonged critical ill patients, as it does in acute sepsis or septic shock [8].

A limitation of this study is reliance of liver biopsy samples taken immediately post-mortem, which is an inherent confounder. However, ethically it was not possible to obtain study-programmed liver samples in unselected critically ill patients. Therefore, findings at the tissue level were always interpreted in the context of serum marker changes from the pre-agonal phase.

In summary, critical illness is associated with a strong increase in serum BA levels. Maintenance of BA-synthesis, suppression of FXR/RXRα, with lowering of apical BSEP and elevated basolateral MRP3 expression may either be a desired response during critical illness to raise serum BA concentrations or it may be a failing feed-back regulation on BA formation and disposition, caused by cholestasis, i.e. increased serum bilirubin and BA.

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Supplemental data

Supplemental table 3.1 Detailed list of assays used for mRNA expression analysis

Gene Name	Gene Symbol	RefSeq	Assay ID
Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	SLC10A1	NM_003049.3	Hs00914889_m1
Solute carrier organic anion transporter family, member 1B1	SLCO1B1	NM_006446.4	Hs01036445_mH
Solute carrier organic anion transporter family, member 1B3	SLCO1B3	NM_019844.2	Hs00991170_m1
ATP-binding cassette, sub-family C (CFTR/MRP), member 3	ABCC3	NM_003786.2	Hs00978471_m1
ATP-binding cassette, sub-family C (CFTR/MRP), member 4	ABCC4	NM_001105515.1	Hs00988706_m1
ATP-binding cassette, sub-family B (MDR/TAP), member 11	ABCC11	NM_003742.2	Hs00994822_m1
ATP-binding cassette, sub-family C (CFTR/MRP), member 2	ABCC2	NM_000392.3	Hs00960494_m1
ATP-binding cassette, sub-family B (MDR/TAP), member 1	ABCB1	NM_000927.3	Hs01067800_m1
ATP-binding cassette, sub-family B (MDR/TAP), member 4	ABCB4	NM_000443.3	Hs00983947_m1
Nuclear receptor subfamily 1, group I, member 3	NR1I3	NM_001077469.1	Hs00901571_m1
Nuclear receptor subfamily 1, group H, member 4	NR1H4	NM_005123.2	Hs00231968_m1
Vitamin D (1,25- dihydroxyvitamin D3) receptor	NR1I1	NM_001017535.1	Hs01045840_m1
Retinoid X receptor, alpha	RXRA	NM_002957.4	Hs00172565_m1
Nuclear receptor subfamily 1, group I, member 2	NR1I2	NM_033013.2	Hs00243666_m1
Cytochrome P450, family 7, subfamily A, polypeptide 1	CYP7A1	NM_000780.3	Hs00167982_m1
Cytochrome P450, family 8, subfamily B, polypeptide 1	CYP8B1	NM_004391.2	Hs00244754_s1
Nuclear receptor subfamily 0, group B, member 2	NR0B2	NM_021969.2	Hs00222677_m1
Hypoxanthine phosphoribosyltransferase 1	HPRT1	NM_000194.1	4310890E

Gene name, Gene symbol, NCBI mRNA reference sequence, and assay ID of the commercial sets of PCR primers and probes (Applied Biosystems, Lennik, Belgium) of the different bile acid transporters and nuclear receptors used for real-time PCR detection.

Supplemental table 3.2 Primary antibodies used for immunohistochemistry and immunoblotting

	Species	Clone		Dilution	Company
CK7	Mouse	OV-TL 12/30	mono	1:400	Dako
NTCP	Rabbit	K9	poly	1:400	*
OATP2/8	Mouse	mMDQ	mono	1:25	Progen Biotechnik
MRP3	Mouse	M_3II-9	mono	1:10	Monosan
BSEP	Rabbit	K12	poly	1:30	*
MRP2	Mouse	M2I-4	mono	1:50	Monosan
MDR1	Mouse	JSB-1	mono	1:10	Monosan
MDR3	Mouse	P3II-26	mono	1:50	Monosan
CAR	Mouse	N4111	mono	1:20	PPMX
FXR	Mouse	A9033A	mono	1:50	PPMX
VDR	Mouse	H4537	mono	1:201	PPMX
RXRα	Mouse	K8508	mono	1:50	PPMX
PXR	Mouse	H-11	mono	1:20	Santa Cruz
CYP7A1	Rabbit	Ab79847	poly	1:200	Abcam
CK18	Mouse	C-04	mono	1:10000	Abcam

Detailed list of all primary antibodies used for immunohistochemistry and immunoblotting, their origin and dilution.
(*) Kindly provided by Bruno Stieger
(Definitions of abbreviations: CK Cytokeratin, NTCP Na^+/taurocholate cotransporting polypeptide, OATP Organic anion transporting polypeptide, MRP Multidrug resistance-associated protein, BSEP Bile salt export pump, MDR Multidrug resistance protein, CAR Constitutive androstane receptor, FXR Farnesoid X receptor, VDR Vitamin D receptor, RXRα Retinoid X receptor alpha, PXR Pregnane X receptor, CYP Cytochrome P450)

Supplemental table 3.3 Baseline characteristics of control and ICU patients

	Control (n=10)	ICU (n=40)	*p-value*
Gender (% male)	70	78	*0.9*
Age (years)	70 ± 5	70 ± 2	*0.9*
BMI (kg/m^2)	25.1 ± 0,8	24.1 ± 0.5	*0.5*
LOS ICU (days)		15 (9-35)	
APACHE II (score)		17 (11-27)	
Diagnostic Group (n,%)			
Cardiovascular disease / high-risk cardiac or complicated vascular surgery		11 ’28)	
Respiratory disease / complicated pulmonary or esophageal surgery		15 (38)	
Gastrointestinal or hepatic disease / complicated abdominal surgery		5 (13)	
Neurology / neurosurgery		3 (8)	
Hematology / oncology		1 (3)	
Solid organ transplant			
Polytrauma		2 (5)	
Renal / metabolic		2 (5)	
Other		1 (3)	
Sepsis (n, %)		19 (48)	
<u>Serum markers on admission</u>			
CRP (mg/L)		100 (61-164)	
ALT (IU/L)		18 (11-35)	
AST (IU/L)		27 (21-57)	
Total Bilirubin (mg/dL)		0.91 (0.51-1.73)	
GGT (IU/L)		43 (27-72)	
ALP (IU/L)		183 (136-249)	
<u>Serum markers on day of biopsy</u>			
CRP (mg/L)	7 (4-28)	149 (73-220)	*<0.0001*
Cytokines			
TNFα (pg/mL)		2705 (9-18500)	
IL-1β (pg/mL)		5230 (77-20030)	
IL-6 (pg/mL)		102895 (292-254970)	
ALT (IU/L)	15 (14-18)	48 (33-73)	*0.0003*
AST (IU/L)	18 (15-21)	53 (36-87)	*<0.0001*
Total Bilirubin (mg/dL)	0.37 (0.29-0.45)	2.01 (1.18-6.83)	*<0.0001*
GGT (IU/L)	25 (20-46)	106 (52-222)	*0.001*
ALP (IU/L)	220 (180-242)	481 (339-692)	*0.0008*
Total bile acids (μM)	0.68 (0.41-1.52)	7.16 (3.48-15.71)	*<0.0001*

Baseline characteristics for 10 control and 40 ICU patients, subset which was used for immunohistochemistry analysis. Represented p-values are calculated for the comparison between ICU and control patients. All data are represented as mean ± SEM or median with IQR (25th-75th percentiles) as appropriate. (Definitions of abbreviations: ICU Intensive care unit, BMI Body mass index, LOS Length of stay, APACHE Acute physiology and chronic health assessment evaluation, CRP C-reactive protein, ALT Alanine aminotransferase, AST Aspartate aminotransferase, GGT Gamma-glutamyltranspeptidase, ALP Alkaline phosphatase, TNFα Tumor necrosis factor alpha, IL Interleukin).

MATERIAL AND METHODS - supplement

Immunoblot analysis of CYP7A1 on liver biopsies

Fifty mg of frozen liver tissue was homogenized with a Precellys 24 machine using ceramic beads (Bertin technologies, Montigny-le-Bretonneux, France) in lysis buffer containing phosphatase inhibitors (Halt Phosphatase Inhibitors Cocktail, Thermo Scientific, Aalst, Belgium). The protein content in the homogenate was determined by the Coomassie Protein Assay Reagent (Thermo Fisher Scientific, Aalst, Belgium). Equal amounts of homogenate proteins (20 µg) were separated by denaturing SDS gel electrophoresis in 10% Bis-Tris polyacrylamide gels (Bio-Rad Laboratories, Nazareth, Belgium) and transferred to nitrocellulose membranes (Hybond, Ammersham Biosciences). Membranes were incubated overnight at 4°C with CYP7A1 primary antibody (Abcam, Cambridge, UK) and goat anti-rabbit HRP-linked secondary antibody (Dako, Heverlee, Belgium) for 1 hour at room temperature. Immunoblots were developed using enhanced chemiluminescence technology (PerkinElmer, Vilvoorde, Belgium) and analyzed using ImageMaster Software 1D Elite (GE Healthcare Europe GmbH, Diegem, Belgium). Data were normalized for cytokeratin 18 (CK18 - Abcam, Cambridge, UK) as a loading control.

Chapter 4:

Impact of parenteral nutrition versus fasting on hepatic bile acid production and transport in a rabbit model of prolonged critical illness

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This chapter is in press:

Vanwijngaerden Y-M, Langouche L, Derde S, Liddle C, Coulter S, Van den Berghe G, Mesotten D. Impact of parenteral nutrition versus fasting on hepatic bile acid production and transport in a rabbit model of prolonged critical illness. *Shock. 2013 Oct 1. [Epub ahead of print]*

4.1. Abstract

Introduction: Cholestatic liver dysfunction frequently occurs during critical illness. Administration of parenteral nutrition (PN) is thought to aggravate this. Underlying mechanisms are not clear.

Methods: In a burn model of prolonged critical illness, rabbits were randomized to a nutritional strategy either accepting caloric deficits (Fasted, n=11) or covering caloric needs by PN (Fed, n=10). At baseline and after 7 days of critical illness, markers of hepatotoxicity, circulating bile acids and the hepatobiliary transport system were studied.

Results: Fasted animals had lower circulating ALT/AST levels than fed animals at day 7. Compared to baseline values, fed animals displayed lower serum unconjugated cholic acid (CA) and deoxycholic acid (DCA) levels. Unconjugated DCA remained unaltered in fasted animals. Unconjugated lithocholic acid (LCA) was increased comparably in all animals, whereas hyodeoxycholic acid (HDCA) was not altered. In contrast, fasting induced a shift from unconjugated CA and DCA to glyco-CA and glyco-DCA. Total bile acids did not correlate with the bile acid producing enzyme CYP7A1, but with the basolateral efflux transporter MRP3. Fasting increased protein expression of the basolateral (MRP3) and the canalicular (BSEP) transporter, whereas the canalicular efflux pump MRP2 was suppressed. Gene expression levels of the nuclear receptor FXR were lower with fasting and correlated inversely with MRP3. The heterodimer partner of FXR, RXRA, was increased with fasting and correlated positively with MRP3.

Conclusion: During prolonged critical illness, withholding PN improved markers for hepatocyte injury and accentuated bile acid transport towards the blood. This suggests that the latter is an adaptive rather than a dysfunctional feedback to illness.

4.2. Introduction

Cholestatic liver dysfunction frequently occurs during critical illness and is associated with poor outcome [1-3]. However, critical illness-related cholestatic liver dysfunction is not yet well characterized and even a clear definition of cholestasis during critical illness is still lacking. Previous work by us and others has indicated that an altered function of the hepatic bile acid transporters may be involved in the pathogenesis of this condition [3-7]. In liver biopsies of critically ill patients, we could demonstrate that the apical bile acid (salt) export pump BSEP was downregulated, whereas the basolateral efflux pumps MRP3 and MRP4, which reflux bile acids back to the circulation, were strongly upregulated. This reversal of bile acid transport to the blood was in line with increased circulating conjugated bile acids and correlated strongly with histopathological markers of bilirubinostasis and ductular reaction [6].

Critical illness is often accompanied by anorexia and a failing of gastro-intestinal function. To prevent caloric deficits, when enteral nutrition is insufficient or poorly tolerated, administration of parenteral nutrition (PN) has been recommended, commencing as early as the first week of critical illness. PN is claimed to play a role in the development of cholestatic liver dysfunction [8] and the mechanisms behind PN-induced cholestasis may include alterations in bile composition and transport as well as direct toxicity by bile acids to the hepatocytes.

In this study we aimed to investigate whether fasting, by withholding PN, limits cholestatic liver dysfunction in a rabbit model of prolonged critical illness. We compared the impact of fasting with PN-feeding during the first week of critical illness on serum levels of ALT, AST and bile acids and correlated these findings with the expression profiles of hepatobiliary transporters and nuclear receptors involved in the regulation of transport and metabolism of bile acids and bilirubin.

4.3. Materials and methods

Experimental study design

The study was performed in a validated rabbit model of prolonged critical illness that comprised the combination of a reproducible third degree burn injury and central vascular access. This model closely mimics the clinical, metabolic and endocrine abnormalities of critically ill patients [9-11]. The model has been described in detail previously [9-11]. In brief, 3-month-old male New Zealand White rabbits were anesthetized and catheters (for blood sampling, intravenous nutrition and insulin administration) were placed under general anesthesia and a full thickness burn injury equaling 15–20% of the total body surface area was inflicted on the flanks after performing a paravertebral block. Next, animals were transferred to individual cages and fluid resuscitation was started with Hartmann solution (Baxter, Lessiness, Belgium) supplemented with 5% glucose (16 mL/h). Blood glucose levels were kept normoglycemic (targeted below 110 mg/dL) with insulin infusion. The animals were deprived from oral feeding but had free access to water and a small amount of hay. On day 1, rabbits were allocated to a fed group receiving a balanced mixed-component parenteral nutrition (84% glucose, 11% protein and 5% lipids) for 6 days (280 kCal/day) or a fasted group that only received dextrose 1.4% with 0.03% NaCl (14 kCal/day). On day 7, animals were sacrificed and samples were taken from liver and snap-frozen in liquid nitrogen. The energy content and composition of the parenteral nutrition is well within the physiological requirement for healthy rabbits. Thereafter, samples were stored at -80°C until further analysis (Figure 4.1). The study was approved by the KU Leuven Ethical Review Board for Animal Research and all animals were treated according to the Principles of Laboratory Animal Care (US National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

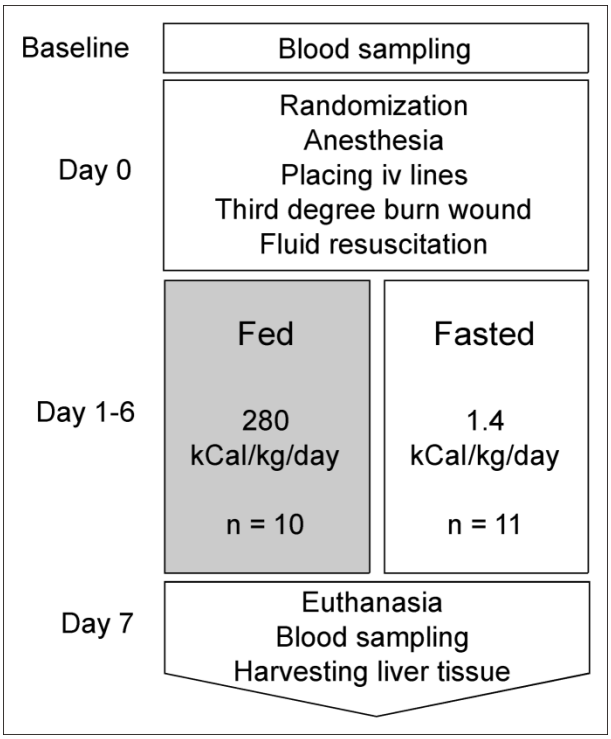

Figure 4.1 Schematic overview of the study protocol

Quantification of serum and liver bile acids levels

Individual serum bile acids were quantified by high performance liquid chromatography-mass spectrometry using authentic bile acid standards and deuterated internal standards. Serum samples (50 µL) were diluted with 50 µL of methanol containing 20.5 ng of deuterated internal standard (cholic acid). The samples were vortexed for 30 seconds, followed by centrifugation at 10,000 rpm for 10 minutes. 150 µL of the supernatant was transferred to a clean Eppendorf tube and dried under nitrogen gas. Samples were reconstituted in 100 µL of the assay mobile phase. Typical recoveries of extracted bile acids exceeded 85%. Chromatographic separations were carried out with a Waters 2695 pump equipped with an autoinjector. The analytes were separated on a Phenomenex Synergi 4µ Hydro-RP 80Å. The mobile phase consisted of solvent A (water), solvent B (methanol), and solvent C (100 mm ammonium acetate, pH 4.5) delivered as a gradient: 0–5 min for solvent B, 55%; 5-15 min for solvent B, 55-75%; 15-23 min for solvent B, 75-80% and 35-40 min for solvent B, 55% with 10% solvent C at a constant flow rate of 0.2 mL/min. The high performance liquid chromatography was coupled with a Waters ZQ quadruple mass detector via an electrospray ionization interface operating in the negative ion mode. Quantitative determination of bile acids was performed by time scheduled single ion recordings using $(M - H)^-$ ions. We determined the optimal parameters for the mass spectrometer for bile acid detection as follows; capillary voltage 3 kV, cone voltage 40 V, extractor voltage 5 V, and RF lens 0.3 V. Source temperature was 100°C and desolvation temperature 300°C. Desolvation gas flow was set at 350 L/h and cone gas flow rate was 60 L/h. The detection limit for individual bile acids was 10 to 50 nmol/L. Total serum and liver bile acid levels were measured enzymatically using a bile acid assay kit in accordance with manufacturer's protocol (Diazyme, Germany). Liver tissue was weighed and homogenized in 75% ethanol and incubated at 50°C for 2h to extract bile acids and centrifuged at 6000g for 10min at 4°C. The bile acid content of the supernatant was determined and normalized with tissue weight used. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by an automated assay using Modular Roche and specific reagents (Roche/Hitachi, Bern, Switzerland).

Cloning of rabbit genes of bile salt transporters and nuclear receptors

For OATP1, OATP8, MRP4, MDR3, CAR (NR1I3), and RXRA (NR2B1) mRNA sequences were not available. Therefore, total RNA was isolated from rabbit liver tissue using Qiazol lysis reagent (Qiagen, Maryland, USA) and subsequently purified using the RNeasy mini RNA isolation kit (Qiagen). cDNA was obtained by reverse transcription of 1 µg total RNA with Super Script III Reverse

Transcriptase (Invitrogen) using random hexamer primers (Invitrogen).Partial coding sequences were synthesized by a PCR procedure using oligonucleotides designed by comparing homology of published sequences from other species. When possible, oligonucleotides surrounded the start and stop codon. Missing 3' ends were cloned by RACE procedure. The amplified fragments were cloned into the pGEM-T vector (Promega) followed by sequence analysis (LGC, Germany). These sequences showed high amino acid identity with the corresponding genes from other mammalian species and data have been submitted to the GenBank database (listed in Supplemental table 4.1). Based upon these sequences, specific primers and probes for real-time PCR analysis were designed using Primer Express software 3.0 (Applied Biosystems, Foster City, CA) and subsequently customized (Eurogentec, Seraing, Belgium).

RNA isolation and real-time PCR

Total RNA was isolated as described above. All liver samples were reverse transcribed simultaneously. Reactions lacking reverse transcriptase were also run as a control for genomic DNA contamination. mRNA levels were quantified in fast real-time PCR with the StepOnePlus platform (Applied Biosystems, Lennik, Belgium) using TaqMan chemistry for accurate quantification of mRNA levels. Sequences of the primers and probes are listed in Supplemental table 4.1. The10 µL real time reaction mixture contained 5 µL TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 0.5 µL forward primer, 0.5 µL reverse primer, 0.5 µL TaqMan probe ([5']6-FAM[3']BHQ-1 labeled), 0.5 µL water, and 3µL cDNA (7.5 ng). Final concentrations were 900 nM for the primers and 300 nM for the probes. Unknown samples were run in duplicate and individual samples with a C_T value standard deviation greater than 0.3 were reanalyzed. Gene expression of ribosomal protein S18 (RPS18) remained stable in all study groups and was therefore used as an internal control. Data are expressed as a fold increase of the mean of the fed group.

Immunoblot analysis

Fifty mg of frozen liver tissue was homogenized with a Precellys 24 machine using ceramic beads (Bertin technologies, Montigny-le-Bretonneux, France) in lysis buffer containing phosphatase inhibitors (Halt Phosphatase Inhibitors Cocktail, Thermo Scientific, Aalst, Belgium). The protein content in the homogenate was determined by the Coomassie Protein Assay Reagent (Thermo Fisher Scientific, Aalst, Belgium). Equal amounts of homogenate proteins (20 µg) were separated by

denaturing SDS gel electrophoresis in 10% Bis-Tris or 3-8% tris-acetate polyacrylamide gels (Bio-Rad Laboratories, Nazareth, Belgium) and separated proteins transferred to nitrocellulose membranes (Hybond, Ammersham Biosciences). Membranes were incubated overnight at 4°C with primary antibody and HRP-linked secondary antibody for 1 hour at room temperature. Further information on the source and dilutions of the primary antibodies for MRP3, MRP4, CYP7A1, CK18 (Abcam, Cambridge, UK), BSEP (Santa Cruz Biotechnology, Santa Cruz, USA), MRP2, and secondary antibodies (DAKO, Denmark) are listed in Supplemental table 4.2. For NTCP, OATP1, OATP8, OSTα, MDR1, and MDR3 no commercially available anti-rabbit antibodies were found. Immunoblots were developed using enhanced chemiluminescence technology (PerkinElmer, Vilvoorde, Belgium) and analyzed using ImageMaster Software 1D Elite (GE Healthcare Europe GmbH, Diegem, Belgium). Data were normalized for cytokeratin 18 (CK18) as a loading control.

Statistical analysis

Statistical analysis was performed using Statview 5.0.1 (SAS Institute, Cary, NC). All quantitative values were assessed for normality. Data were presented as mean ± SEM or medians with IQR (1^{st}-3^{rd}) when appropriate. Paired measurements of the circulating bile acids were analyzed with Wilcoxon Signed Rank tests. Differences among study groups were analyzed by unpaired t-tests for normally distributed data and by Mann-Whitney U tests for non-normally distributed data. Correlations were calculated using Pearson tests. For all tests a *p*-value less than 0.05 was deemed significant.

4.4. Results

Circulating levels of ALT, AST and bile acids

After 7 days of critical illness, serum levels of the AST and ALT were lower in fasted compared to PN-fed critically ill rabbits (Figure 4.2). In contrast, the levels of total bile acids in the serum were not different between PN-fed and fasted critically ill rabbits (Figure 4.2). However, critical illness induced a shift in the composition of the circulating bile acid pool (Figure 4.3). In rabbits, unconjugated cholic acid (CA), deoxycholic acid (DCA), lithocholic acid (LCA) and hyodeoxycholic acid (HDCA) are the main circulating bile acids. If conjugated, they are predominantly glycine-bound. Median healthy baseline levels of CA were 0.14 µM (IQR 0.07 – 0.36), of DCA were 7.59 µM (IQR 3.62 – 18.52), of LCA were 0.38 µM (IQR 0.18 - 0.78), of HDCA were 2.76 µM (IQR 1.63 – 4.48), of G-CA were 0.16 (IQR 0.05 – 0.21) and of G-DCA were 0.34 (IQR 0.12 – 0.86). Compared to baseline values, PN-fed rabbits displayed lower unconjugated CA and DCA levels, whereas in fasted rabbits only unconjugated DCA levels were not altered. Unconjugated LCA was increased comparably in both PN-fed and fasted critically ill rabbits, whereas HDCA was unaffected by critical illness. In contrast, conjugated glyco-deoxycholic acid (G-DCA) was increased in fasted critically ill rabbits, but not in PN-fed rabbits. This shift from unconjugated to conjugated bile acids in fasted animals was reflected in a 10- to 15-fold increase of the G-CA/CA ratio and G-DCA/DCA ratio in fasted rabbits. PN-fed rabbits displayed a 3-fold increase in the G-CA/CA ratio (Figure 4.3).

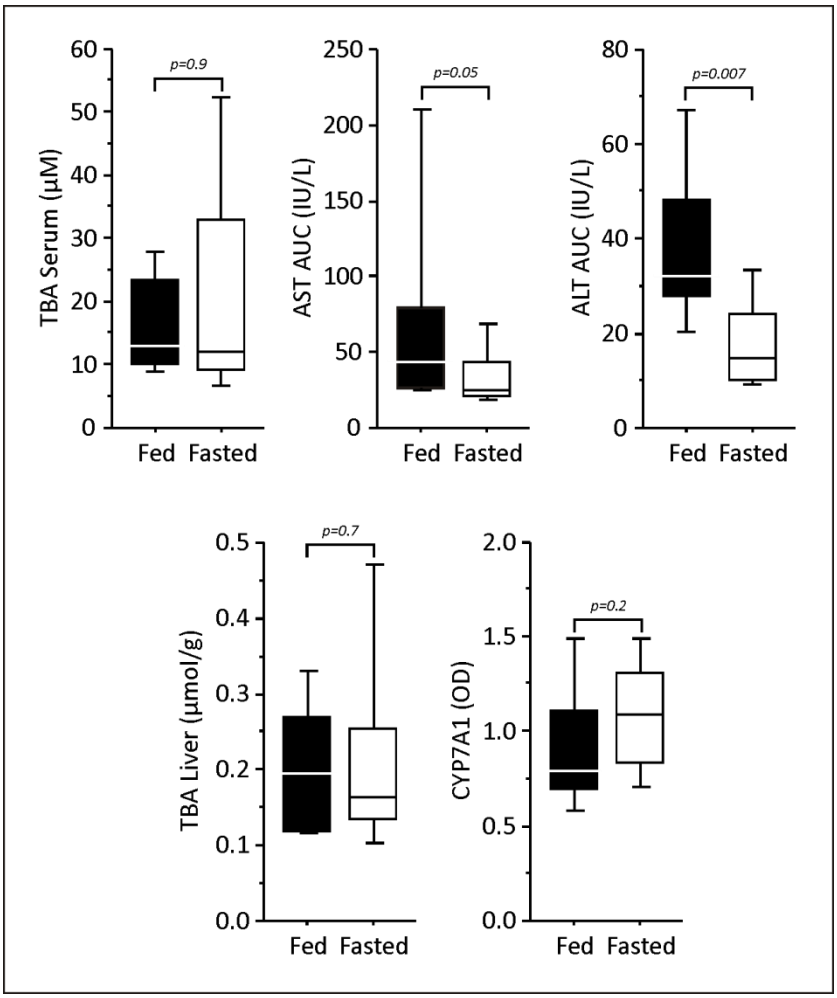

Figure 4.2 Serum TBA, ALT and AST and liver TBA and CYP7A1 in PN-fed and fasted prolonged critically ill rabbits

TBA serum shows day 7 circulating total bile acid concentration, ALT and AST AUC is the area under the curve using daily measurements, TBA liver and CYP7A1 shows the day 7 values quantified in liver homogenates. Median healthy baseline levels were 13.0 µM (IQR 7.5-18.9) for TBA, 41 U/L (IQR 31-61) for AST, and 30 U/L (IQR 18-61) for ALT. Levels are expressed as median with IQR (25th-75th percentiles). Abbreviations: TBA, total bile acids; AUC, area under the curve; OD, optical density.

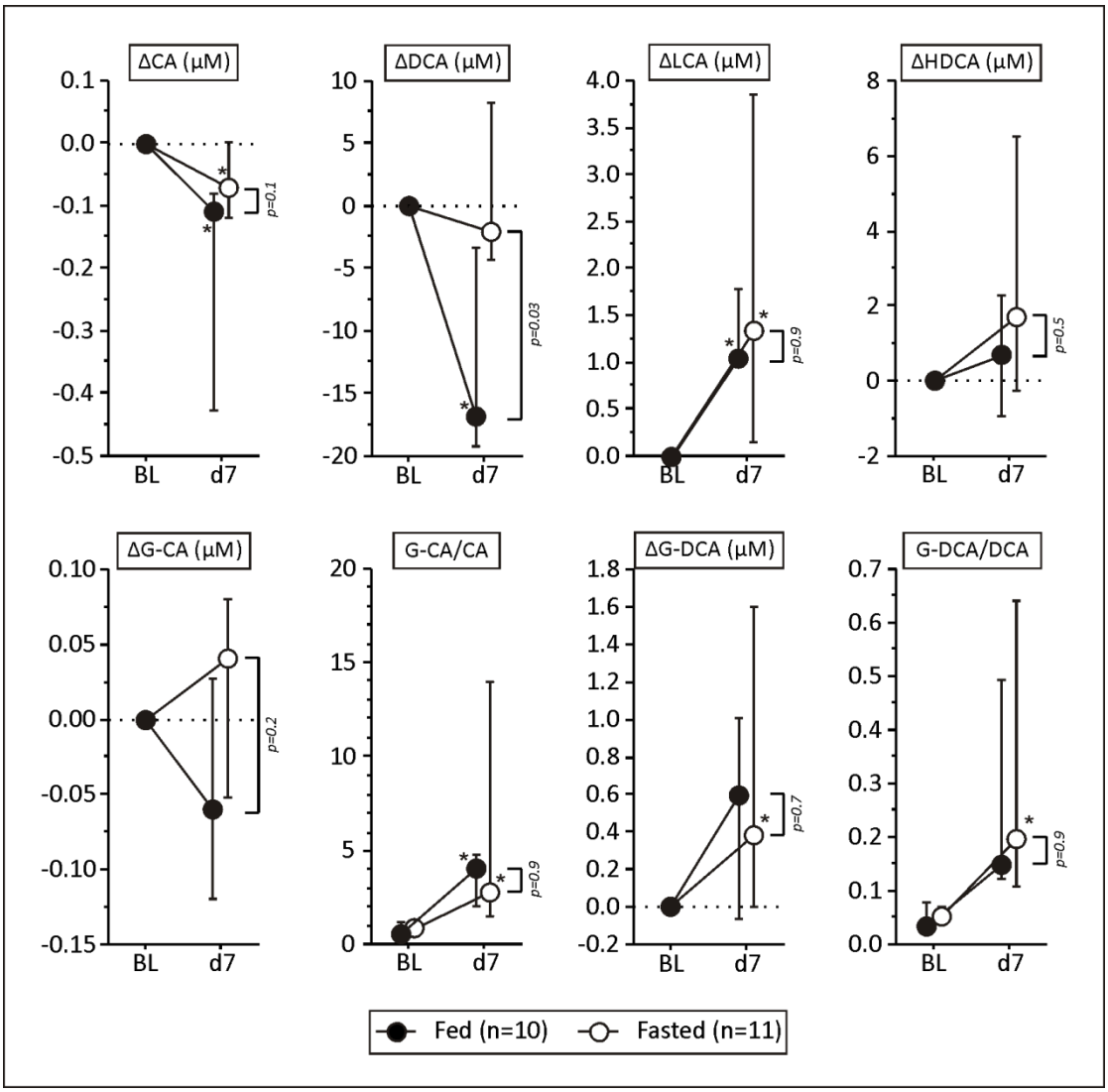

Figure 4.3 Circulating bile acid concentrations in TPN-fed and fasted critically ill rabbits

Bile acids were determined by mass spectrometry at baseline (BL) and day 7 (d7) after onset of the illness and are expressed as the delta change from baseline. Levels are expressed in μM and are represented as median with IQR. * represents p≤0.05 for comparison of changes over time (baseline levels versus day 7 levels) using the Wilcoxon Signed Rank test. The p-values depicted on each panel were calculated using the nonparametric Mann-Whitney U test for comparison of the differences over time between the fed and the fasted animals. Abbreviations: CA, cholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; HDCA, hyodeoxycholic acid; G-CA, glycocholic acid; G-DCA, glycodeoxycholic acid.

Bile acid synthesis enzymes and hepatobiliary transporters

Hepatic protein levels of CYP7A1, the rate limiting enzyme in the bile acid synthesis, were not different between TPN-fed and fasted critically ill rabbits (Figure 4.2). Protein levels correlated well

with mRNA levels of CYP7A1 (r=0.515; *p=0.02*). No correlation between the serum levels of total bile acids and hepatic mRNA or protein CYP7A1 levels was observed.

Hepatic protein levels of the basolateral bile acid efflux transporters MRP3 and MRP4 were significantly higher in fasted compared with PN-fed critically ill rabbits (Figure 4.4). MRP3 and protein levels correlated inversely with mRNA levels (r=-0.553, *p=0.009*) and MRP4 protein levels did not correlate with mRNA levels. Gene expression of OSTα was not altered (Supplemental figure 4.1).

We could not quantify protein levels of the basolateral uptake transporters NTCP, OATP1 and OATP8, but mRNA levels of NTCP (*p=0.0003*) and OATP8 (*p=0.002*) were lower in fasted compared with PN-fed critically ill rabbits (Supplemental figure 4.1).

Hepatic protein levels of the canalicular efflux pump MRP2 were lower in fasted compared with PN-fed critically ill rabbits (Figure 4.4). MRP2 protein levels correlated positively with MRP2 mRNA levels (r=0.871, *p<0.0001*). In contrast, the canalicular efflux pump BSEP protein levels were higher in fasted compared with PN-fed critically ill rabbits. However, BSEP protein levels did not correlate with BSEP mRNA levels. We could not quantify protein levels of the canalicular efflux pumps MDR1 and MDR3, but gene expression levels were lower in fasted compared with TPN-fed critically ill rabbits (*p=0.0007* for MDR1, *p=0.0004* for MDR3, Supplemental figure 4.1).

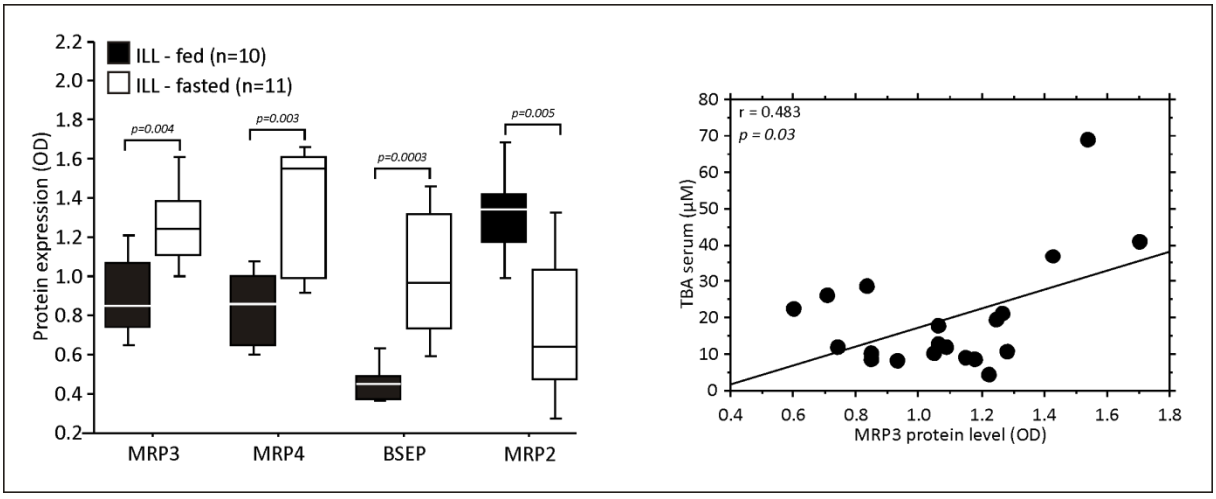

Figure 4.4 Protein levels of hepatobiliary transporters in PN-fed and fasted prolonged critically ill rabbits

Hepatic basolateral efflux transporters (MRP3, MRP4), and canalicular efflux pumps (BSEP, MRP2) were quantified by Western blot using liver homogenates. Data are represented as median with IQR (25th-75th percentiles). Abbreviations: MRP, multidrug resistance-associated protein; BSEP, bile salt export pump, OD optical density.

Nuclear receptors

To identify the upstream regulators of the transporter expression levels, we quantified gene expression of the key regulating nuclear receptors. In normal physiological conditions farnesoid X receptor (FXR) is the predominant bile acid sensor, but also retinoid X receptor alpha (RXRA), the vitamin D receptor (VDR), pregnane X receptor and constitutive androstane receptor (CAR) are involved in the regulation of BA metabolism and/or transport [12].

Gene expression levels of the nuclear receptors FXR and CAR were significantly lower in fasted compared with PN-fed critically ill rabbits (Figure 4.5). mRNA levels of FXR correlated inversely with the protein levels of the basolateral efflux pumps MRP3 and MRP4 and positively with the protein levels of the canalicular efflux pump MRP2 (Figure 4.5). Gene expression of the nuclear receptor RXRA was higher in fasted compared with PN-fed critically ill rabbits and correlated positively with MRP3 protein levels (Figure 4.5). Gene expression of the nuclear receptors PXR and VDR did not differ between the 2 study groups.

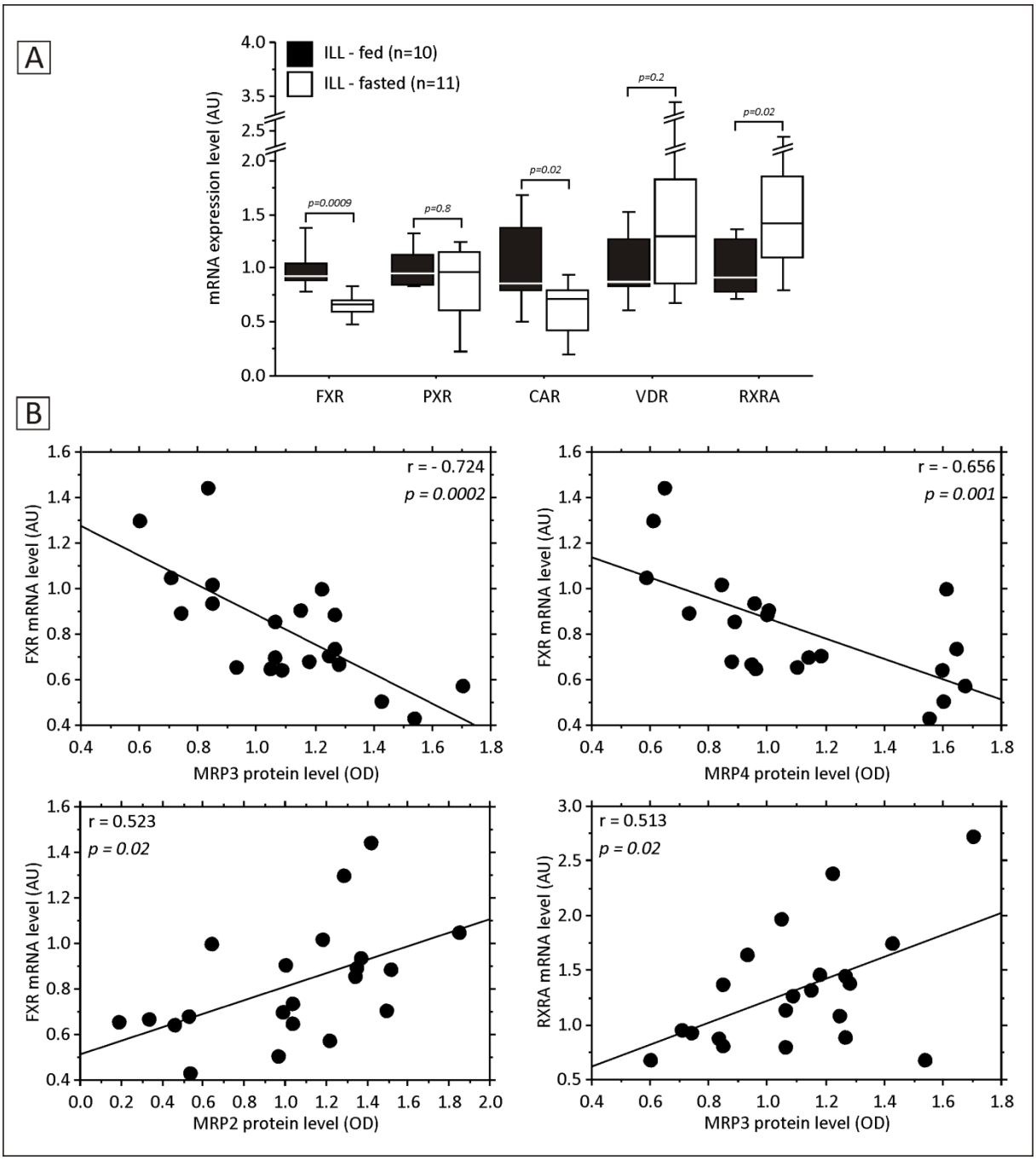

Figure 4.5 (A) Gene expression levels of nuclear receptors in PN-fed and fasted prolonged critically ill rabbits. (B) Correlations between nuclear receptors and hepatobiliary transporters

Data in (A) are represented as median with IQR (25th-75th percentiles). Abbreviations: FXR, farnesoid X receptor; VDR, vitamin D receptor; CAR, constitutive androstane receptor; PXR, pregnane X receptor; RXRA, retinoid X receptor alpha. AU, arbitrary units, MRP multidrug resistance-related protein.

4.5. Discussion

This study in a rabbit model of prolonged critical illness found that fasting by withholding PN reduced markers of hepatocellular injury and induced a shift towards more conjugated and less toxic bile acids. Fasting during critical illness was also associated with an increased protein expression of the bile acid efflux transporters at the basolateral (MRP3) and apical (BSEP) membranes. The increased expression of MRP3 was strongly associated with suppression of FXR.

Fasting during prolonged critical illness in rabbits resulted in decreased levels of AST and ALT, indicating suppressed parenchymal damage. Parenchymal liver damage during critical illness, also called hypoxic liver injury [13], is associated with poor outcome in the ICU [14]. This is in contrast with the findings from a randomized controlled trial in adult critically ill patients [15]. Here, withholding PN during the first week of critical illness did not affect the number of patients with a clinically important increase in levels of AST and ALT. Surprisingly, fasting by withholding PN resulted in a higher proportion of patients with hyperbilirubinemia during the first week of critical illness. In the critically ill rabbits we could not detect bilirubin in the serum, neither by the conventional enzymatic essays nor by High Performance Liquid Chromatography. For this reason we focused on the bile acids as markers of cholestasis. Withholding PN did not affect the concentration of total bile acids in the serum or in the liver. Nevertheless, fasting induced a shift towards more conjugated bile acids. This indicates a protective response as conjugated bile acids are less toxic than their unconjugated counterparts. In critically patients the unconjugated bile acids, cholic acid and chenodeoxycholic acid, did not differ from controls, but went together with a large increase in the concentration of conjugated bile acids [6]. Similarly, the change in bile acid concentration could not be explained by increased de novo bile acid synthesis as the protein expression level of CYP7A1 was unaltered [6].

Hence, changes in the transport of the bile acids between the hepatocyte, the sinusoidal blood compartment and the canalicular bile compartment are most likely controlling the altered bile acid composition during critical illness. The strong upregulation of the basolateral efflux transporters MRP3 and MRP4 by fasting is striking. Under normal conditions these transporters exhibit only low expression levels in hepatocytes. Prolonged critical illness [6], inflammation [16] and longstanding cholestasis [17] have been associated with increased MRP3 and MRP4 expression. It is presumed that that the basolateral efflux transporters reverse the transport of primarily conjugated bile acids from the canalicular bile compartment to the blood for subsequent renal elimination. The observations that withholding PN reduced markers of hepatocellular injury in combination with increased MRP3

and MRP4 expression suggests that MRP3/4 upregulation is a protective response, at least from the standpoint of the liver. Whether the increased levels of bile acids and bilirubin, and the shift towards more conjugated bile acids in critically ill patients [6] and animals [18] holds a survival benefit is not clear. It suggests that the association between the increase of bilirubin levels during the first 48 hours after ICU admission and the risk of mortality described in an observational study [1] may not be causal. However, in other studies serum bilirubin levels were found to be a poor discriminator of increased mortality risk during critical illness [19;20].

In observational studies during critical illness, artificial nutrition has been associated with the development of liver dysfunction [21;22]. The EPaNIC randomized controlled trial demonstrated that withholding PN lead to fewer patients with a clinically important increase in levels of GGT and ALP during the entire ICU stay, suggesting less cholestatic liver dysfunction [15]. Nevertheless, the proportion of patients with hyperbilirubinemia was higher in the late PN group. As this patients group had a shortened ICU stay, less new infections in the ICU and a faster recovery from organ failure, one can question whether hyperbilirubinemia alone is an appropriate marker of poor ICU outcome.

The metabolism and transport of the bile acids is tightly regulated by a complex network of nuclear receptors, of which FXR is the most important. Critical illness leads to decreased levels of FXR and its heterodimeric partner RXRA in the nucleus of the hepatocytes [6]. Fasting during critical may lead to a further decrease in the levels FXR. However, the gene expression levels of RXRA were increased by withholding PN in this rabbit study of critical illness. As we previously described discrepant responses between the gene and protein expression level, interpretation of the data should be done with caution [6]. However, taken that withholding PN during critical illness holds a beneficial response, reduced expression of the bile acid sensor FXR, maintenance of bile acid synthesis (CYP7A1) and reversal of bile acid transport (MRP3) may constitute a protective response. Indeed, previous studies in bile duct ligated mice have demonstrated that genetic abrogation of Fxr reduces liver injury and improves survival, accompanied by strong upregulation of the basolateral BA efflux transporter Mrp4 [23;24], a direct parallel of what was observed in the present study. This suggests that FXR antagonists may be therapeutically useful across a ranges of conditions where bile acids contribute to liver injury.

Alternatively, the changes in bile acid metabolism and transport may reflect a dysfunctional feedback system and may herald poor outcome [20]. Starvation in non-critical care conditions gives rise to exacerbated liver apoptosis in FXR knock-out mice [25].

This study has some limitations. First, extrapolation from an animal model of critical illness to the human context is difficult. This was further complicated by the difficulty encountered in measuring bilirubin levels and protein levels of the basolateral bile acid transporters OATP and NTCP. Secondly, although animals were randomized into two groups, the association between the bile acids, their transporters and the regulating nuclear receptors cannot delineate causality. Future studies in which those components of bile acid regulation are manipulated by overexpression and knock-out models could provide additional mechanistic insight. Thirdly, sham-operated, pair-fed animals were not added to the study. This was done as the key question was the impact of parenteral nutrition versus nutrient restriction during critical illness.

In conclusion, during prolonged critical illness, withholding PN improved markers for hepatocellular injury in association with the reversal of normal bile acid trafficking and increased bile acid detoxification though conjugation. This suggests that fasting in critical illness induces adaptive changes in bile acid homeostasis, changes that could possibly be emulated by therapeutic interventions targeting FXR [12;26].

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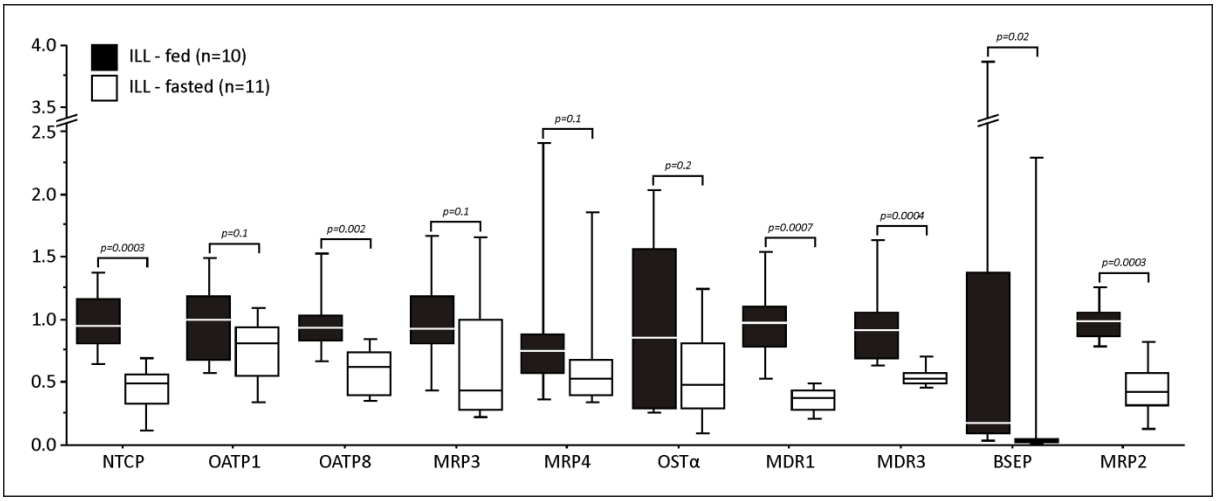
Supplemental data

Supplemental figure 4.1 Gene expression of hepatobiliary transporters in PN-fed and fasted critically ill rabbits.



Hepatic basolateral influx pumps (NTCP, OATP1, OATP8), basolateral efflux transporters (MRP3, MRP4, OSTα), and canalicular efflux pumps (BSEP, MRP2, MDR1, MDR3). mRNA levels are expressed in arbitrary units relative to the mRNA expression of the housekeeping gene RPS18 and relative to 10 fed critically ill rabbits. Data are represented as median with IQR (25^{th}-75^{th} percentiles). Abbreviations: NTCP, Na^+/taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; MRP, multidrug resistance-associated protein; OST, organic solute transporter; BSEP, bile salt export pump; MDR, multidrug resistance protein.

Supplemental table 4.1 Primers and probes for real-time PCR gene expression analyses

Name	NCBI Accession number	Sequence 5'-Fw primer-3' 5'-Rv primer-3' 5'-Probe-3'
NTCP	NM_001082768.1	5'-TCTCTGCCCTGATGCCTTTT-3' 5'-CGTGCACTAAGGCGGAAGA-3' 5'-TGGCTTCCTGCTGGGCTTCATTCTCT-3'
OATP1	KC708581	5'-CTTTTTGATTTGTGCAGGAGTGA-3' 5'-GATTATCCTCTAGTCCTTCCTTTGGA-3' 5'-TGTGCTCACTGCCATCCCTTTTTTCTTT-3'
OATP8	KC708580	5'-GAATGGTATGGAAGCAATAATGGAT-3' 5'-GGATGCTCGATGGGTTGGT-3' 5'-ATTCCAGCCATAAGGAAACCAAGCCACC-3'
MRP3	AY289920.1	5'-GCAATTACTCAGAGGAGGACATCTG-3' 5'-GCTGCGCCCTCACGAA-3' 5'-CAAGCCCTGGAGCTGGCCCAC-3'
MRP4	KC708579	5'-TTGCAAGCAAAATCATCGTGTT-3' 5'-CACGGCTGGCTGTGATGAC-3' 5'-ACCTTCACAGTCTATGTGCTCCTGGGCAA-3'
OSTα	DQ122755.1	5'-GTGCTCACCAGGAAGAAGCTTAA-3' 5'-GCGAGATCTTGAAGAACGCATA-3' 5'-CTGCTGATGTTGGGCCCATTCCA-3'
MDR1	NM_001082159.1	5'-AACTGGAAGGTTCCGGAAAGA-3' 5'-CTTCTGCTCACGAGTCAGAGACA-3' 5'-TGCCACAGAAGCAATAGAGAACTTCCGAA-3'
MDR3	KC708578	5'-TTCTGGTGTTCTCAGCAATCGT-3' 5'-GCGTAGTCCGGAGCAAAGG-3' 5'-CGGTGGCTCTCGGACACGCC-3'
BSEP	NM_001082083.1	5'-GAGGGAACTACCAGGACAGTTTAAGA-3' 5'-TGCCAGGTAAGAAAGCTGAGACT-3' 5'-CGTCTCTCCGGCAGCGCTCC-3'
MRP2	Z49144.1	5'-GCTTTCCCATGAGCATGCTT-3' 5'-GCCGATCCACGGAAACACT-3' 5'-CCAATGTGATCTCCGCCATGCTCC-3'
FXR NR1H4	NM_001082726.1	5'-CAAGATTCATCAGCCCGAGAA-3' 5'-AACGTCCGCAGCTCTGTCA-3' 5'-CCTCAACACTTCGCCTGCCTCCTG-3'
CAR NR1I3	KC708577	5'-TCAGACGAACGGTCAATAAATACC-3' 5'-GGCCCTGCTGACCTCACA-3' 5'-TCTCACCTGCCCCTTTGCTGG-3'
PXR NR1I2	NM_001082067.1	5'-GCTGACAGAAGTGGGAAAAAGC-3' 5'-ATGCCTTTGAACATGTAGGTTGAC-3' 5'-TTTTCTCTGCTGCCCCACTTGGCTG-3'
VDR NR1I1	AY262279.1	5'-AGCAGCAGCGCATCATTG-3' 5'-GGCATAGGTAGGGTCGTAGGTCTT-3' 5'-CATCCTGCTGGACGCCCACCA-3'
RXRA NR2B1	KC708576	5'-ATGGCCAGGCACTTCTGGTA-3' 5'-AAGGACTGCCTGATCGACAAG-3' 5'-CGGCAGTACTGGCACCGGTTCC-3'
CYP7A1	NM_001170929.1	5'-CAGGGACCACATCTCAGAACTG-3' 5'-CGTCGAAGGTGGAGAGTGTGT-3' 5'-TCCGCCTGCGCATGTTTCTGAA-3'
RPS18	KC708528	5'-TTCCGCATGATGGTGATCAC-3' 5'-GCAGACATTGACCTGACCAAGA-3' 5'-CGTTCCACCTCGTCCTCAGTGAGCTCT-3'

Supplemental table 4.2 Primary and Secondary Antibodies for immunoblotting

Antibody	Species	Clone	Dilution	Company
MRP3	Mouse	M2II-21 (Ab3376)	1:50	Abcam
MRP4	Mouse	M4I-10 (Ab15602)	1:50	Abcam
BSEP	Mouse	F-6 (Sc-74500)	1:100	Santa Cruz
MRP2	Guinea Pig	C-terminal	1:1000	Gift*
CYP7A1	Rabbit	C-terminal (Ab79847)	1:200	Abcam
CK18	Mouse	C-04 (ab668)	1:10000	Abcam
Anti-mouse IgG/HRP	Goat	P0447	1:1000	Dako
Anti-rat IgG/HRP	Rabbit	P0450	1:1000	Dako
Anti-rabbit IgG/HRP	Goat	P0448	1:1000	Dako
Anti-guinea pig IgG/HRP	Rabbit	P0141	1:1000	Dako

Abbreviations: MRP, multidrug resistance-associated protein; BSEP, bile salt export pump; CK, cytokeratin; Ig immunoglobulin; HRP horse radish peroxidase. * MRP2 antibody was a kind gift from Prof. F.G.M. Russel, University of Nijmegen, The Netherlands [1].

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Chapter 5:

Withholding parenteral nutrition during critical illness increases plasma bilirubin but lowers the incidence of biliary sludge

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This chapter is in press:

Vanwijngaerden Y-M, Langouche L, Brunner R, Debaveye Y, Gielen M, Casaer M, Liddle Ch, Coulter S, Wouters PJ, Wilmer A, Van den Berghe G, Mesotten D. Withholding parenteral nutrition during critical illness increases plasma bilirubin but lowers the incidence of biliary sludge. *Hepatology. 2013 Nov 9. [Epub ahead of print]*

5.1. Abstract

Cholestatic liver dysfunction (CLD) and biliary sludge often occur during critical illness and are allegedly aggravated by parenteral nutrition (PN). Delaying initiation of PN beyond day 7 in ICU (late PN) accelerated recovery as compared with early initiation of PN (early PN). However, the impact of nutritional strategy on biliary sludge and CLD has not been fully characterized.

This was a preplanned subanalysis of a large RCT of early PN versus late PN (n=4640). In all patients plasma bilirubin (daily) and liver enzymes (ALT/AST/GGT/ALP; twice weekly; n=3216) were quantified. In a random predefined subset of patients also plasma bile acids (BAs) were quantified at baseline and on days 3, 5 and last ICU day (n=280). Biliary sludge was ultrasonographically evaluated on ICU day 5 (n=776).

From day 1 after randomization until the end of the 7-day intervention window, bilirubin was higher in the late PN than in the early PN group ($p<0.001$). In the late PN group, as soon as PN was started on day 8, bilirubin fell and the two groups became comparable. Maximum levels of GGT, ALP and ALT were lower in the late PN group ($p<0.01$). Glycine/taurine-conjugated primary BAs increased over time in ICU ($p<0.01$), similarly for the two groups. Fewer patients in the late PN than in the early PN group developed biliary sludge on day 5 (37% vs 45%; $p=0.04$).

In conclusion, tolerating substantial caloric deficit by withholding PN until day 8 of critical illness increased plasma bilirubin but reduced the occurrence of biliary sludge and lowered GGT, ALP and ALT. These results suggest that hyperbilirubinemia during critical illness does not necessarily reflect cholestasis and instead may be an adaptive response that is suppressed by early PN.

5.2. Introduction

Cholestatic liver dysfunction (CLD) – most typically defined as hyperbilirubinemia above 3 mg/dL - occurs in almost 20% of critically ill patients and is an independent risk factor for unfavorable outcome [1-3]. Hyperbilirubinemia is a common phenotype of numerous diseases and syndromes in critically ill patients. Mechanical obstruction of the extrahepatic bile duct is easily and robustly diagnosed by ultrasonography, but is only a rare cause of CLD in the intensive care unit (ICU). CLD is predominantly due to intrahepatic non-obstructive cholestasis, which is essentially the inability of the hepatocyte to secrete bile into the bile duct, leading to an intracellular accumulation of bilirubin and bile acids (BAs). Although increased concentrations of BAs inside the hepatocytes are the likely cause of cholestatic liver damage, plasma concentrations of bilirubin, and/or alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) are mostly used in clinical practice for CLD diagnosis [4].

Biliary sludge may also be part of the spectrum of CLD during critical illness due to a lack of bile flow. Notably, critically ill patients with absent oral intake and on total parenteral nutrition are susceptible to the development of biliary sludge. This also applies to patients after gastric surgery or transplantation and patients in whom antibiotics such as ceftriaxone are used [5]. The clinical relevance of gallbladder sludge is however unclear and therapeutic interventions are reserved for patients who develop acalculous cholecystitis.

Parenteral nutrition (PN) is assumed to aggravate both CLD and biliary sludge formation [6]. In a large randomized controlled multicenter trial we have recently assessed the outcome effect of tolerating a nutritional deficit during the first week in ICU (Late PN), compared with early initiation of PN to supplement insufficient enteral feeding (early PN) [7, 8]. Late PN enhanced organ function recovery, reduced the incidence of new infections and shortened duration of ICU stay. Strikingly, more patients in the late PN group had hyperbilirubinemia above 3 mg/dL, whereas fewer late PN patients had a clinically relevant increase in levels of GGT or ALP [8]. We therefore hypothesized that increased bilirubin levels during critical illness do not necessarily reflect onset of clinically significant CLD. In contrast, hyperbilirubinemia may represent an adaptive response, as bilirubin can act as an endogenous anti-oxidant and may counteract the pro-oxidative effects of BA [9-11].

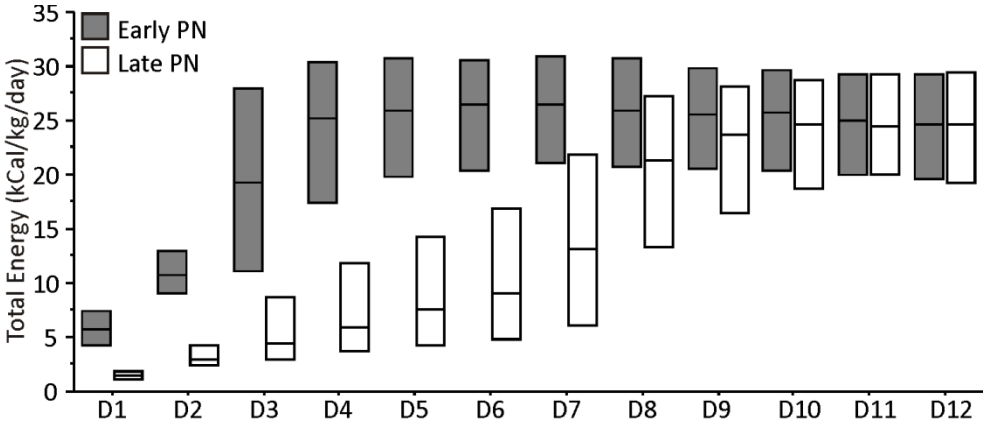
Therefore, the aim of the present study was to compare the effect of late versus early PN on circulating bilirubin, BAs and the liver enzymes GGT, ALP, alanine aminotransferase (ALT), aspartate

aminotransferase (AST) over time. In addition, we evaluated the impact of late PN versus early PN on biliary sludge by ultrasonography on ICU day 5.

5.3. Material and methods

Study design and patient characteristics

This study was a preplanned prospective subanalysis of a large randomized controlled trial on the effect of early versus late initiation of PN on the outcome of critical illness (EPaNIC trial) [12]. The study protocol was previously described in detail [8]. Patients were allocated either to the early PN group, where PN was initiated within 48 hours after ICU admission to supplement insufficient enteral nutrition (early PN n=2312) or to the late PN group, where PN was not initiated before day 8 (late PN n=2328) (Figure 5.1). Baseline characteristics for both allocation groups were comparable in the total study population (Supplemental table 5.1).



	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
Early PN	2312	1833	1438	1185	975	848	736	646	601	562	517	479
Late PN	2328	1885	1399	1117	913	760	655	574	524	481	436	397

Figure 5.1 Total energy intake levels during the EPaNIC study

Boxes represent daily total caloric intake (glucose, lipids and proteins) expressed as medians with IQR (25^{th}-75^{th} percentiles). The grey boxes represent daily caloric intake of patients randomized to receiving early parenteral nutrition (early PN), whereas open boxes are presenting values from patients randomized to nutrient restriction (late PN). The number of patients still in ICU is plotted for each day. Abbreviations: IQR interquartile range, PN parenteral nutrition, ICU intensive care unit.

Time course of bilirubin was assessed in all patients while in ICU (early PN n=2312; late PN n=2328). Liver enzymes ALT, AST, GGT and ALP were quantified twice weekly in all patients. Additionally, in 3216 EPaNIC patients (early PN n=1760; late PN n=1794) also plasma conjugated bilirubin was quantified during ICU stay as part of the daily routine clinical practice.

For determination of the plasma levels of conjugated and unconjugated bile acids and conjugated bilirubin, a subgroup of patients, identifiable upon ICU admission, was chosen. Only patients for whom early enteral nutrition was surgically contra-indicated were selected [8]. This selection was chosen to prevent possible bias by enterohepatic recycling of BA and bilirubin by enteral feeding. Of the 517 patients in this subgroup (256 patients in the early PN group and 261 patients in the late PN group) a random selection of 280 patients (140 out of each allocation group) was made to reduce the number of samples for analysis, while testing the hypothesis with enough statistical power. Random selection was performed by a computer algorithm (StatView software, SAS Institute Inc., Cary, North Carolina). Patients in the early and late PN group were comparable for baseline characteristics (Supplemental table 5.1). In this subgroup, nutritional intake was according to the study protocol (Supplemental figure 5.1).

For analysis of the impact of early versus late PN on gallbladder sludge, all patients who were still in ICU on the morning of day 5 (early PN n=975, late PN n=913) were eligible for ultrasonography of the gallbladder (Figure 5.2). Patients with a history of cholecystectomy (n=90), patients who were moribund (n=2), or patients who were discharged from ICU on day 5 before 12 am (n=147) were excluded from the ultrasonography study. Additionally, 783 patients were excluded due to logistical reasons (such as unavailability of ultrasonography device or ultrasound assessor). In total 776 patients (early PN n=420; late PN n=356) underwent ultrasonographical investigation of the gallbladder on day 5 of their ICU stay. Baseline characteristics in both treatment groups were comparable (Supplemental table 5.1).

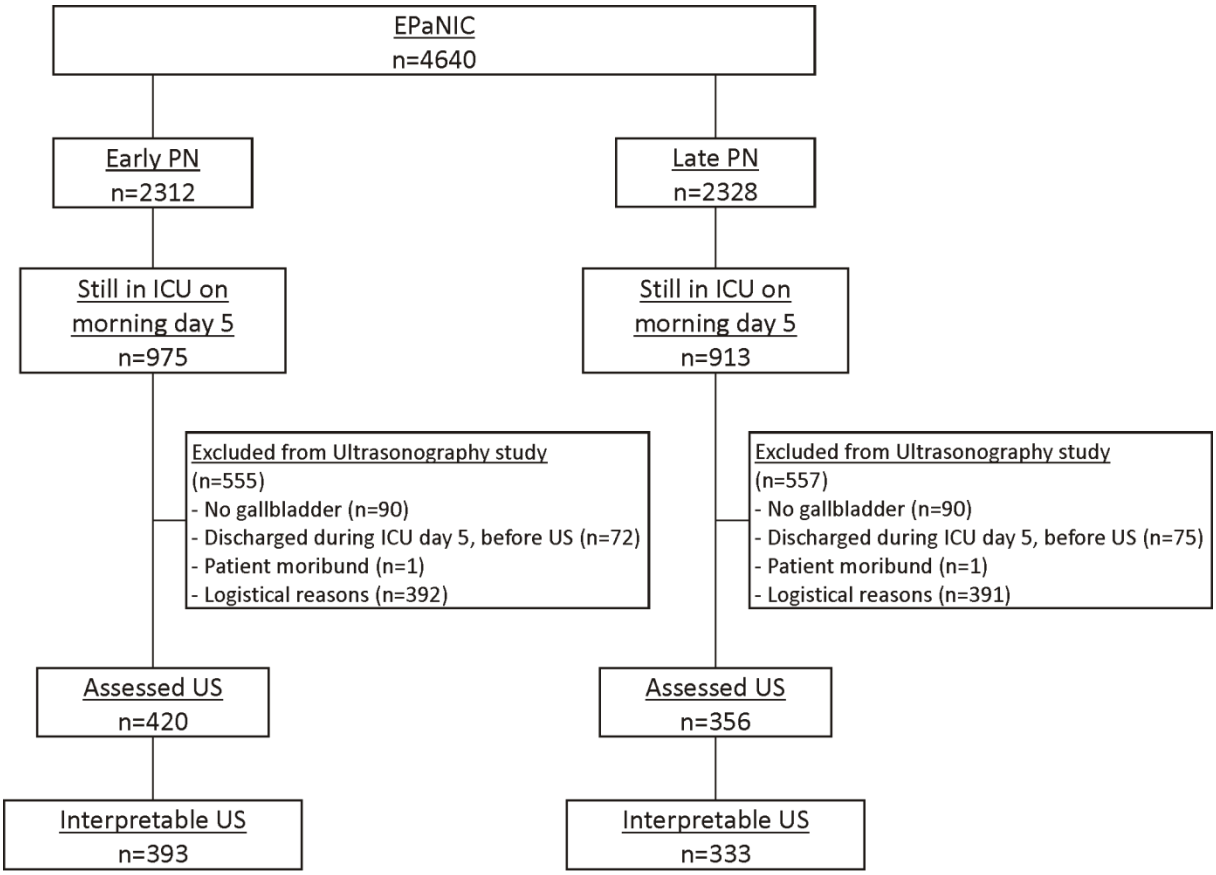


Figure 5.2 Consortdiagram of enrollment for the ultrasonography study

Only patients with an ICU stay of at least 5 days were selected for evaluation. Abbreviations: PN parenteral nutrition, ICU intensive care unit, US ultrasonography.

Plasma concentrations of bilirubin, liver enzymes and bile acids

Plasma total and conjugated bilirubin were quantified by a standard routine automated laboratory assays (colorimetric DPD-method, HITACHI/Roche for Cobas c702). Liver enzymes ALT, AST, GGT and ALP were quantified by standard routine automated laboratory assays (ALT and AST with the UV kinetic method according to the International Federation of Clinical Chemistry (IFCC), GGT with the Szasz kinetic colorimetric assay and ALP with an AMP kinetic colorimetric assay according to the IFCC, all Hitachi/Roche for Cobas c702).

Plasma concentrations of unconjugated and conjugated primary bile acids cholic acid and chenodeoxycholic acid were measured, as well as the conjugated and the unconjugated secondary bile acid deoxycholic acid by high performance liquid chromatography-mass spectrometry by using authentic bile acid standards and deuterated internal standards, as previously described [13].

Ultrasonography of the gallbladder

Gallbladder sludge, wall thickness and wall doubling were evaluated by blinded assessors (YV, YD, MG) using ultrasonography. The diagnosis of gallbladder sludge was based on the presence of a low-amplitude echogenic collection layering in the most dependent portion of the gallbladder. A thickened gallbladder wall (> 5mm) and wall doubling (presence of pericholecystic fluid) were assessed at the anterior part of the gallbladder.

Statistical analysis

Statistical analysis was performed with Statview 5.0.1 (SAS Institute Inc., Cary, North Carolina SAS). All quantitative values were assessed for normality. Values with normal distribution, and those that were normalized after logarithmic transformation, were compared with the unpaired and paired Student's t-test. The non-normally distributed data were compared by the non-parametric Mann-Whitney U-test and Wilcoxon signed rank test. Nominal and ordinal variables (expressed as numbers and percentages) were compared with Fisher's exact test. Correlations between variables were calculated using the Pearson's rank correlation test. For all tests a p-value less than 0.05 was deemed significant. To assess the potential predictive power of circulating bilirubin and/or bile acids, area under the receiver operator characteristics curve (AROC) values were calculated with SPSS software (SPSS version 2.0, IBM Corp, New York, USA). Additionally, the relationship between categorized day 1 bilirubin levels and mortality of all EPaNIC patients was plotted.

5.4. Results

Effect of late initiation of parenteral nutrition on bilirubin and the other liver enzymes tests

Peak plasma levels of total bilirubin determined during the whole ICU stay and determined during the study intervention window (first 7 days in ICU) were higher in late PN patients in comparison with early PN patients (Table 5.1). From day 1 after randomization until the end of the 7-days intervention window, also daily plasma total bilirubin was higher in the late PN than in the early PN group (all *p<0.001*) (Figure 5.3). From day 8 onwards, when PN was commenced in late PN patients and total caloric intake levels became comparable in both groups, plasma total bilirubin was no longer different between late and early PN patients. In the subgroup of patients admitted with sepsis (early PN n=510, late PN n=505) changes were comparable to those in the total population: bilirubin levels were higher in late PN patients from day 2 until day 8 (*p<0.05*) and became similar in the two groups from day 8 onwards. Conjugated bilirubin levels quantified during clinical routine in 3216 EPaNIC patients correlated with total bilirubin (r>0.900 and *p<0.01* for all) and were higher in late PN compared to early PN patients (*p<0.05* until day 7). Also, peak levels of conjugated bilirubin were higher in late versus early PN patients (Table 5.1). In the total patient population, conjugated levels represented 80% of total bilirubin.

Table 5.1 Effect of early versus late parenteral nutrition on peak plasma concentrations of total bilirubin and liver enzymes

	ICU D1-LD			ICU D1-D8		
	Early PN	**Late PN**	***p-value***	**Early PN**	**Late PN**	***p-value***
T Bilirubin (mg/dL)	0.91 [0.60-1.64]	1.01 [0.67-1.78]	*< 0.0001*	0.87 [0.59-1.48]	0.97 [0.65-1.66]	*< 0.0001*
C Bilirubin (mg/dL)	0.39 [0.21-1.01]	0.45 [0.24-1.16]	*0.0003*	0.37 [0.20-0.85]	0.43 [0.24-1.02]	*< 0.0001*
GGT (IU/L)	50 [19-140]	38 [18-115]	*0.0007*	40 [18-99]	35 [17-84]	*0.002*
ALP (IU/L)	178 [102-373]	159 [99-332]	*0.02*	155 [100-275]	149 [97-257]	*0.1*
ALT (IU/L)	28 [16-79]	24 [14-65]	*0.0003*	23 [14-51]	21 [14-45]	*0.005*
AST (IU/L)	55 [34-106]	55 [33-104]	*0.3*	50 [32-87]	50 [32- 92]	*0.9*

Peak plasma concentrations of total bilirubin and liver enzymes for the total EPaNIC study ICU stay and for the EPaNIC study intervention time window (first 8 days of ICU stay) are presented as median [IQR]. P-values are calculated with Mann-Whitney U test. Abbreviations: ICU intensive care unit, C conjugated, T total, GGT gamma-glutamyl transferase, ALP alkaline phosphatase, ALT alanine aminotransferase, AST aspartate aminotransferase

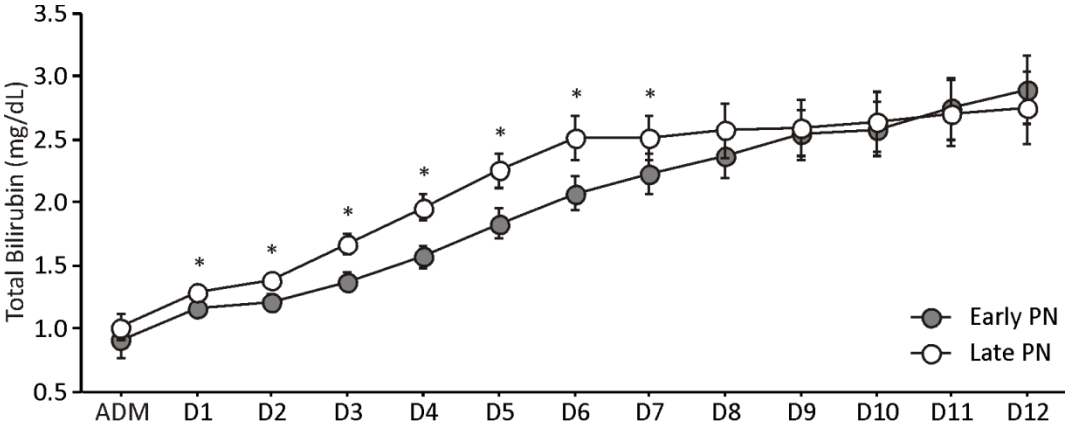


Figure 5.3 Daily plasma total bilirubin levels

Daily plasma total bilirubin levels of all patients still in ICU are presented as mean ± standard error of the mean. The grey dots present values of early PN patients, whereas open dots are presenting values from late PN patients. * *p≤0.05* with the unpaired Student's t-test after logarithmic transformation. Abbreviations: PN parenteral nutrition, ADM admission.

In contrast with bilirubin, peak levels of GGT were lower in the late PN group compared with the early PN group determined during the total ICU stay as well as during the study intervention window (Table 5.1). Peak levels of ALP were also lower in the late PN group in comparison with the early PN group, but only when determined over the total ICU stay. Peak levels of the hepatocyte lysis enzyme ALT were lower in the late PN group compared to the early PN group during the total ICU stay as well as during the study intervention window. Peak levels of AST did not differ between the 2 treatment groups (Table 5.1).

Effect of late initiation of parenteral nutrition on bile acids and conjugated bilirubin

In the subset of patients for whom early enteral nutrition was surgically contra-indicated, circulating BAs were determined by mass-spectrometry. In these patients, compared with the admission values, the circulating glycine- and taurine-conjugated primary BAs, cholic acid and chenodeoxycholic acid, were elevated on day 3 and day 5 of ICU stay (Figure 5.4). Also, the unconjugated primary BA cholic acid was mildly increased on day 3 and day 5. Remarkably, the concentration of conjugated cholic acid was 5- to 50-fold higher than the levels of unconjugated cholic acid (*p<0.01* for all – Figure 5.4). The secondary BA, deoxycholic acid, either unconjugated or conjugated, did not change over time in these critically ill patients. The levels of circulating bile acids were comparable in early and late PN patients. In this subgroup of patients, conjugated bilirubin levels correlated well with the total

bilirubin levels on admission, on day 3 and day 5. They were higher in the late PN patients compared to the early PN patients (Figure 5.5). Of the total bilirubin levels, 35% was conjugated.

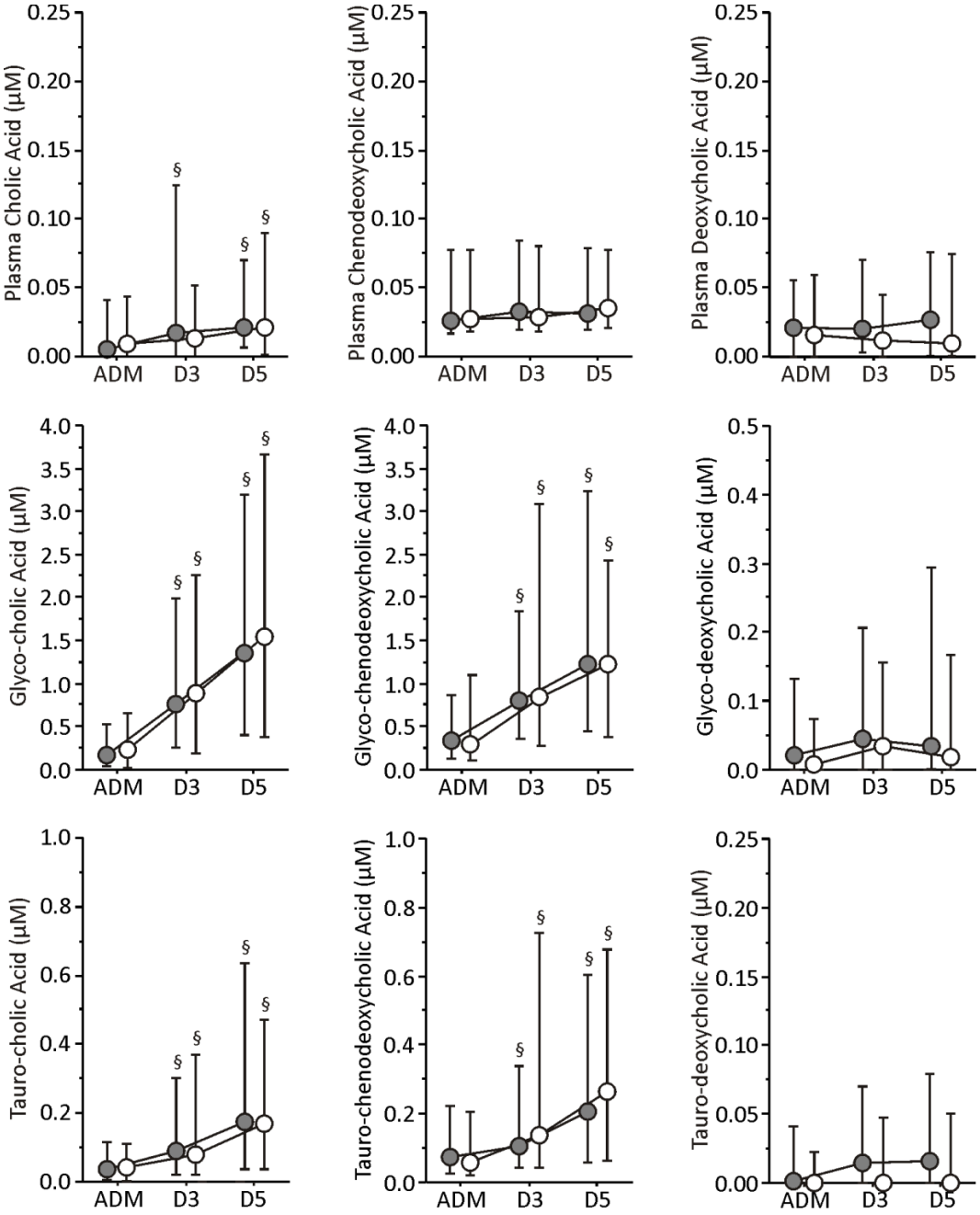


Figure 5.4 Circulating plasma levels of bile acids

Plasma levels of bile acids (expressed in µM) on admission, on day 3 and day 5 of ICU stay are represented as median with IQR (25^{th}-75^{th} percentiles). The grey dots present values of early PN patients (n=140), whereas open dots are presenting values from late PN patients (n=140). § *p≤0.05* using Wilcoxon rank test for comparison with admission values. Abbreviations: ADM admission, IQR interquartile range, PN parenteral nutrition.

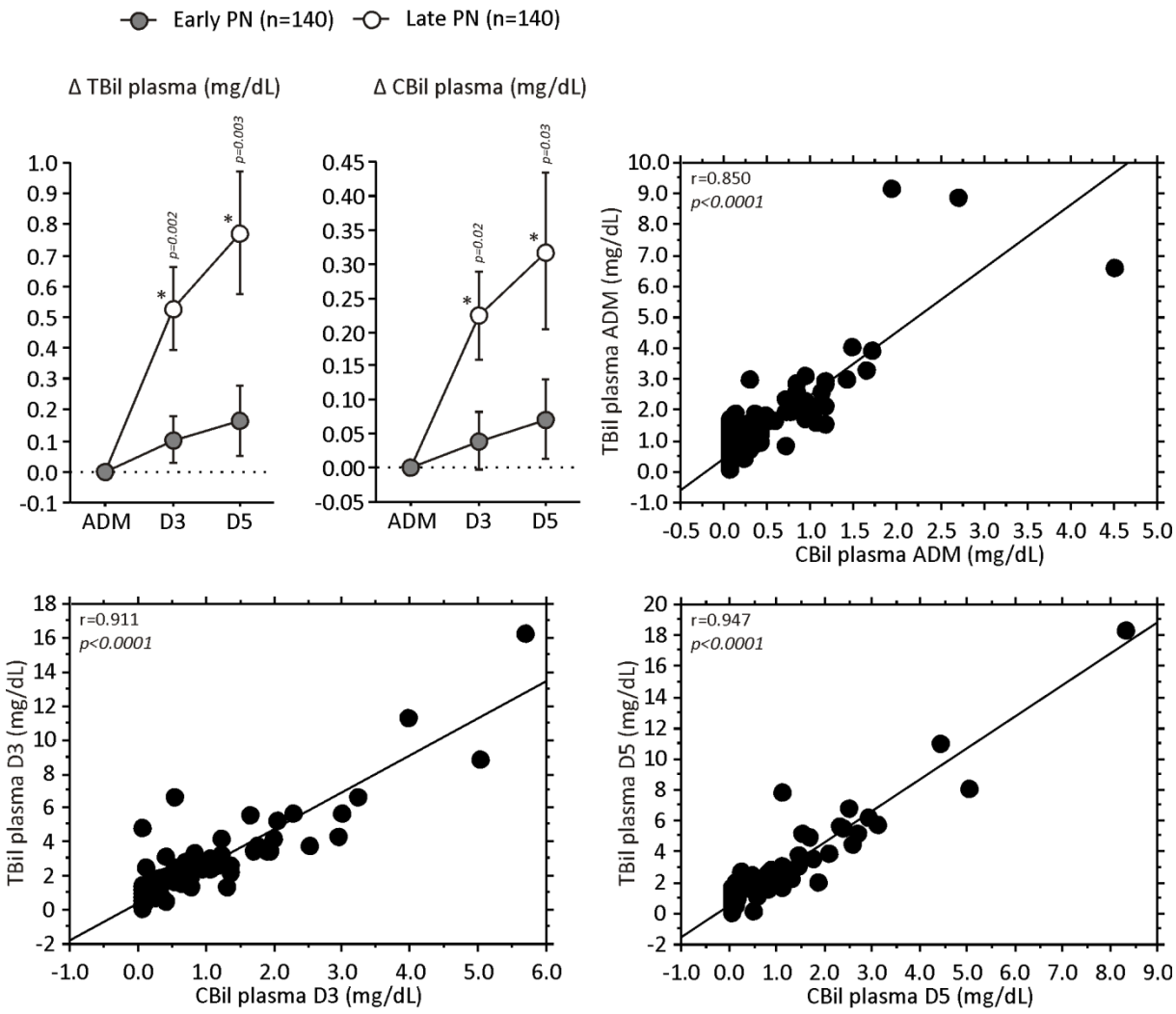


Figure 5.5 Effect of early versus late initiation of parenteral nutrition on total and conjugated bilirubin levels

The line charts (mean ± SEM) represent the changes from the admission values (referred to as Δ) to day 3 in ICU and to day 5 in ICU in plasma total bilirubin and conjugated bilirubin. The grey dots represent the values of early PN patients, whereas the open dots are presenting values from late PN patients. * p≤0.05 using the Wilcoxon signed rank test for calculations of changes over time; p-values for calculations of differences between early and late PN values obtained using Mann-Whitney U test. Correlations between plasma total bilirubin levels and conjugated bilirubin levels on admission, on day 3 and day 5 of ICU stay are calculated using Pearson's correlation test. Abbreviations: SEM standard error of the mean, ICU intensive care unit, TBil total bilirubin, CBil conjugated bilirubin, ADM admission.

Effect of late initiation of parenteral nutrition on gallbladder sludge

Fewer patients in the late PN group developed gallbladder sludge than in the early PN group (37% versus 45%; *p=0.04*)(Table 5.2). Also the incidence of double gallbladder wall tended to be lower in the late PN group in comparison with the early PN group (3.3% versus 6.1%, *p=0.08*). The incidence of wall thickening was comparable for both groups (5.7% versus. 6.2%, *p=0.8*).

Table 5.2 Ultrasonography study of the gallbladder

	Early PN (n=420)	**Late PN (n=356)**	***p-value***
Sludge – n(%)	175 (44.8)	124 (37.3)	*0.04*
Wall thickening - n(%)	24 (6.2)	19 (5.7)	*0.8*
Double wall - n(%)	24 (6.1)	11 (3.3)	*0.08*

Predictive value of bilirubin and bile acids for ICU mortality

The relationship between day 1 bilirubin levels and ICU mortality displays a 'hockey stick' shape (Figure 5.6): very low levels of bilirubin (<0.36 mg/dL) were associated with a mild increase in mortality risk while patients with normal to mildly elevated bilirubin levels (0.36 – 2.39 mg/dL) displayed the lowest mortality rates. Only in patients with day 1 bilirubin exceeding 2.40 mg/dL was the ICU mortality risk sharply increased. The AROC curve value for day 1 bilirubin to predict ICU mortality was 0.597 (calculated in the total study population of 4640 patients). The AROC values of admission BAs to predict ICU mortality ranged from 0.455 (tauro-deoxycholic acid) to 0.631 (tauro-chenodeoxycholic acid), calculated in the bile acid study population (n=280).

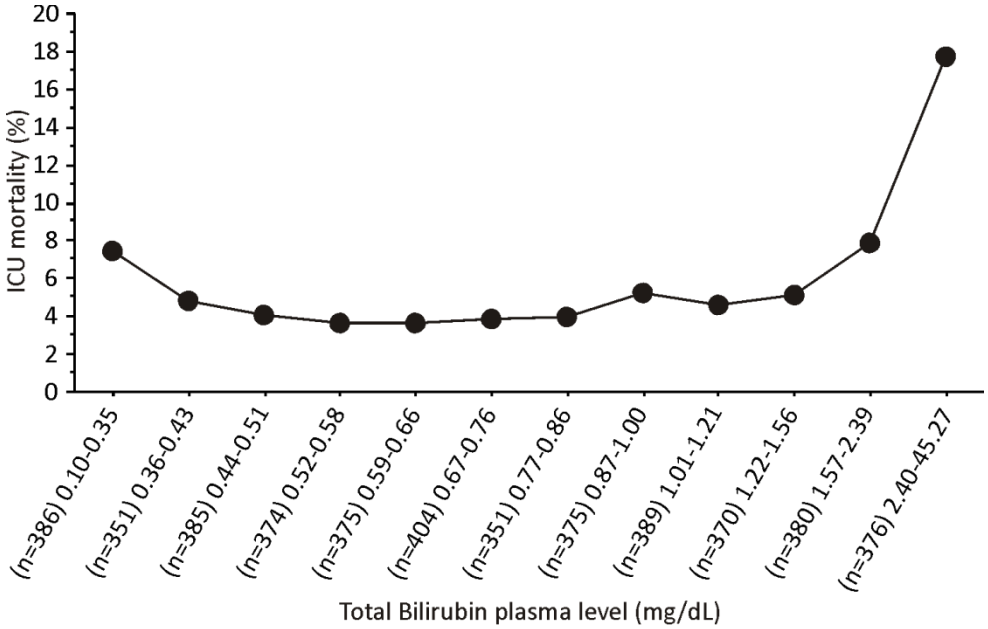


Figure 5.6 Relationship between plasma total bilirubin levels on day 1 and ICU mortality

5.5. Discussion

This study demonstrated that early initiation of parenteral nutrition in critically ill patients immediately suppressed plasma bilirubin concentrations. This effect of early PN occurred without affecting the plasma concentrations of circulating bile acids. Early PN during critical illness increased the levels of cholestatic liver enzymes GGT and ALP and increased the incidence of biliary sludge. The latter is in line with the previously observed association between long-term administration of PN and the development of biliary sludge in critically ill patients [14] and with the results from a rabbit experiment documenting gallbladder distension within 1 week of PN [15]. However, the association between PN and biliary sludge in the critical care setting has always been biased by the fact that more severely ill patients do not tolerate enteral feeding and rely on PN to maintain caloric intake. With the current study, a causal relationship was established. We demonstrated that postponing the administration of PN reduced the incidence of gallbladder sludge during critical illness. Biliary sludge thus appears to be in part a preventable complication of critical illness as metabolic interventions such as tolerating caloric deficits by late PN but also tight blood glucose control [2] synergistically lowered the occurrence of gallbladder sludge. Whether the caloric restriction is bringing about this effect directly or indirectly through the decreased incidence of ICU acquired infections [8] and lowered antibiotic use [16] cannot be delineated from this study.

We previously reported that avoiding early administration of PN during the first week lowered the proportion of patients with GGT and ALP levels above 1.5 x upper limit of normality (ULN), our a priori definition of cholestasis during ICU stay [2, 8]. While average peak levels of GGT and ALP were higher in the early versus late PN group, peak levels still remained below this 1.5 x ULN cut-off. Similar subtle differences were seen in plasma ALT levels. While mean levels were higher in the early PN group, the proportion of patients with ALT > 3 x ULN did not differ between the treatment groups [8]. Taken together, this indicates that overt cholestasis, as seen in chronic TPN administration and hyperalimentation, is not frequent in critically ill patients. However, early administration of PN may not be well tolerated by the liver during critical illness, causing a relatively mild elevation of liver enzymes [6].

In contrast with the liver enzymes, peak total bilirubin levels, the archetypical biochemical marker of cholestasis during critical illness, remained lower in the patients exposed to the early administration of PN. Conversely, patients in the late PN group consistently revealed higher bilirubin levels coinciding with a better outcome (shorter ICU stay and less ICU acquired infections). As soon as PN was started after 1 week in the late PN group, this difference was mitigated. It confirms our previous finding that ‘cholestasis’ defined by bilirubin levels > 3 x ULN is more frequent in the late PN group

during the intervention window [8]. Hence, a rise in plasma bilirubin may be an adaptive response in the context of caloric restriction during critical illness. Also, in other population studies elevated bilirubin levels had a protective effect against coronary artery disease [17] and stroke [18]. Hyperbilirubinemia may exert its beneficial effect by improving endothelial function and decreasing oxidative stress [19]. Heme oxygenase-1, which is the rate-limiting enzyme in the bilirubin production, is protective in endothelial cells against toxicity associated with high glucose and oxidative stress [20]. Heme oxygenase knock-out mice have been demonstrated to have a higher mortality and more liver and kidney injury during endotoxic shock [21]. Additionally, bilirubin administration was shown to attenuate liver and kidney damage in animal models [22, 23].

However, in this study peak bilirubin levels were associated with ICU mortality risk in a 'hockey stick' relationship. Very low levels of bilirubin are associated with a mild increase in mortality risk. Hyperbilirubinemia on admission only correlated with increased mortality risk when levels exceed 2.5 -3.0 x ULN. This may explain why bilirubin, analyzed as a continuous variable, has a poor predictive power for mortality in non-cirrhotic critically patients [24]. Nevertheless, peak bilirubin levels are still used in the most prevalent scoring systems for organ failure such as the SOFA [25], MOD [26] and SAPS score [27]. Importantly, peak plasma concentration of bilirubin or GGT/ALP do not seem interchangeable in the definition of overt cholestasis [2, 6, 28]. GGT/ALP may be the better indicators for cholestasis during critical illness, as these enzymes are believed to reflect 'cholate stasis' in the bile canaliculi [29]. Alternatively, a higher cut-off for bilirubin as a marker of CLD should be clinically validated.

We also observed that the bile acid profile was affected by critical illness, but not by the timing of PN initiation. In contrast to Recknagel et al. [24], in our study population, plasma concentration of BAs did not predict mortality of critically ill patients better than did plasma bilirubin. The conjugated primary bile acids, cholic acid and chenodeoxycholic acid, increased over time while the concentrations of unconjugated bile acids were not significantly altered during the course of critical illness. Also the conjugated secondary bile acid, deoxycholic acid, was unchanged. This lack of impact on circulating BAs may partially be explained by the selection of patients with a surgical contraindication to enteral feeding. Consequently, the enterohepatic BA cycle may have been impaired. Whether enteral nutrition would exert an additional effect on circulating BAs, cannot be addressed as the study was not randomized for enteral nutrition and the specific subset of patients for whom BAs were quantified, did not receive any enteral nutrition. Similarly, in the subset of patients with a contraindication to enteral feeding, the proportion of conjugated bilirubin was much lower than in the overall patient population. The mechanism of this conjugation deficit is not clear

and may be related to the higher severity of illness observed in the patient population with a contra-indication to enteral nutrition. However, unconjugated bilirubin may have more potent anti-oxidative properties [30]. Therefore, the mild unconjugated hyperbilirubinemia early in the course of critical illness may be a different entity to the pronounced conjugated hyperbilirubinemia typical of prolonged critically ill patients, the so called 'ICU jaundice' [31]. Nevertheless, the dual role of bilirubin during critical illness remains associative from our study data. Only an interventional study, in which circulating levels of bilirubin are actively manipulated, can answer whether mild hyperbilirubinemia truly protects the patient during critical illness.

In conclusion, withholding PN and accepting a large caloric deficit during the first week of critical illness increased plasma concentrations of bilirubin, lowered plasma levels of GGT and ALP but not of BAs, and reduced the incidence of gallbladder sludge. These results suggest that hyperbilirubinemia during critical illness does not necessarily reflect cholestasis. Instead hyperbilirubinemia may be an adaptive response, which is suppressed by early PN.

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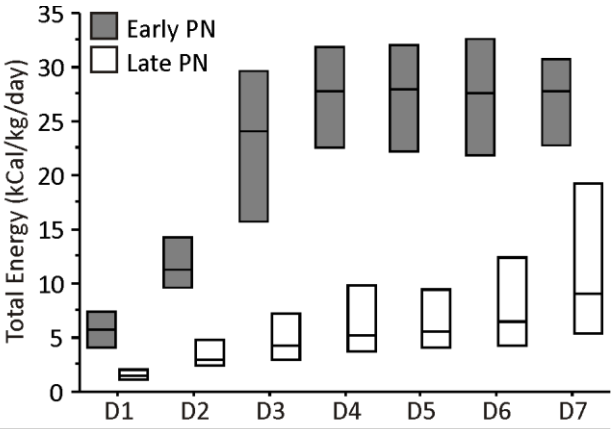
Supplemental data



Early PN	140	121	109	100	92	86	77
Late PN	140	121	96	88	80	70	68

Supplemental figure 5.1 Total energy intake levels during the EPaNIC study for the subgroup of patients selected for analysis of bile acids

Boxes represents daily total caloric intake (glucose, lipids and proteins) expressed as medians with IQR (25^{th}-75^{th} percentiles). The grey bars represent daily caloric intake of patients randomized to receiving early parenteral nutrition (early PN), whereas open bars are presenting values from patients randomized to nutrient restriction (late PN). The number of patients still in ICU is plotted for each day. Abbreviations: IQR interquartile range, PN parenteral nutrition, ICU intensive care unit.

Supplemental table 5.1 Baseline characteristics

Patient characteristic	Total population			Subgroup bile acids			Subgroup ultrasonography		
	Early PN n=2312	Late PN n=2328	*p-value*	Early PN n=140	Late PN n=140	*p-value*	Early PN n=420	Late PN n=356	*p-value*
Male sex — n (%)	1486 (64)	1486 (64)	*0.8*	87 (62)	87 (62)	*>0.9*	280 (67)	230 (65)	*0.5*
Age - yr	64 ± 14	64 ± 15	*0.5*	63 ± 14	62 ± 15	*0.7*	63 ± 15	63 ± 16	*0.8*
Weight - kg	76 ±16	75 ± 15	*0.05*	74 ±17	73 ± 17	*0.8*	75 ±15	75 ± 16	*0.6*
BMI – kg/m^{2-} n(%)			*0.3*			*0.5*			*0.6*
<20	134 (6)	141 (6)		12 (9)	20 (14)		28 (7)	27 (8)	
20 to <25	854 (37)	890 (38)		65 (46)	55 (39)		172 (4)	139 (39)	
25 to <30	852 (37)	864 (38)		41 (29)	42 (30)		136 (32)	129 (36)	
30 to <40	430 (19)	405 (17)		19 (14)	21 (15)		75 (18)	57 (16)	
≥40	42 (2)	28 (1)		3 (2)	2 (1)		9 (2)	4 (1)	
Diabetes mellitus – n (%)	391 (17)	417 (18)	*0.4*	17 (12)	19 (14)	*0.7*	65 (15)	58 (16)	*0.8*
Malignancy - n (%)	437 (19)	457 (20)	*0.9*	86 (61)	83 (59)	*0.7*	97 (23)	84 (24)	*0.9*
NRS score	4 [3-4]	4 [3-4]	*0.3*	4 [3-5]	4 [3-5]	*0.4*	4 [3-5]	4 [3-5]	*0.9*
Sepsis - n (%)	510 (22)	505 (22)	*0.8*	80 (57)	68 (49)	*0.2*	189 (45)	171 (48)	*0.4*
Emergency admission - n (%)	956 (41)	970 (42)	*0.8*	89 (64)	92 (66)	*0.7*	306 (73)	266 (75)	*0.6*
APACHE II score	23 ± 11	23 ± 10	*0.8*	27 ± 11	27 ± 11	*0.7*	31 ± 10	31 ± 9	*0.9*

Baseline characteristics of the patients of the total study population, the subgroup of patients selected for analysis of bile acids and the subgroup of patients who underwent ultrasonographical evaluation of the gallbladder. Scores from the Acute Physiology and Chronic Health Evaluation II (APACHE II) reflects severity of illness and can range from 0 to 71, with higher scores indicating a greater severity of illness [32]. Scores from Nutritional Risk Screening (NRS) range from 0 to 7, with higher scores indicating a higher risk of malnutrition [33]. NRS scores are presented as medians with IQR between square brackets. Plus-minus values are means ± SD. Percentages may not total 100 because of rounding. Abbreviations: BMI body mass index, SD standard deviation, IQR interquartile range.

Chapter 6:

General discussion and conclusions

Up to 20% of the patients in the intensive care unit develop cholestatic liver dysfunction (CLD), which has been associated with an increased risk of mortality [1-3]. Cholestasis can be defined as a decrease in bile flow due to either obstruction of bile flow through mechanical obstruction of the hepatic bile ducts or to an inability of the hepatocyte to secrete bile into the bile duct. Mechanical obstruction of the extrahepatic bile duct is easily and robustly diagnosed by ultrasonography, but is only a rare cause of CLD in critically ill patients. In contrast, critical illness related CLD is predominantly due to intrahepatic non-obstructive cholestasis, leading to an intracellular accumulation of bilirubin and bile acids.

Although increased concentrations of bile acids inside the hepatocytes are presumed to induce cholestatic liver damage, the molecular and biochemical changes underlying CLD are poorly characterized and clear diagnostic criteria are lacking. The most widely used definition of CLD in clinical practice is a hyperbilirubinemia above 3 mg/dL. However, as a causal link between hyperbilirubinemia and worse outcome is missing, it may even be a biochemical epiphenomenon. Furthermore, hyperbilirubinemia might represent an adaptive response, as bilirubin can act as an endogenous anti-oxidant and may counteract the pro-oxidative effects of BA [4-6]. Additionally, the reliability of hyperbilirubinemia as a marker of cholestasis in critically ill patients may be questionable, since there are many factors that can influence the levels of bilirubin.

In this thesis, we therefore aimed at further understanding the cholestatic changes at biochemical and molecular level during critical illness. The central hypothesis of this doctoral research project states that ‘cholestasis’, defined as ‘hyperbilirubinemia’ in the early phase of critical illness is brought about by changes in bile acid synthesis and transport and is a protective response of the liver. As parenteral nutrition (PN) is assumed to aggravate both CLD and biliary sludge formation [7], we further postulate that parenteral nutrition could modify this protective cholestatic response in the early phase of critical illness.

As explained above, whether hyperbilirubinemia reflects truly cholestasis during critical illness is unclear. Therefore, the aim of the first study was to examine a large cohort of critically ill patients to gain mechanistic insights into ICU-jaundice, with a focus on bile acids (BAs), hepatocytic transporters involved in bile production as well as their regulating nuclear receptors (Figure 6.1).

We studied circulating levels of BAs and bilirubin levels in 130 non-surviving ICU and 20 control patients of which liver biopsies were available. In the liver biopsies levels of BAs synthesis enzymes, BAs transporters and nuclear receptors were assessed and a histological evaluation of cholestasis was performed.

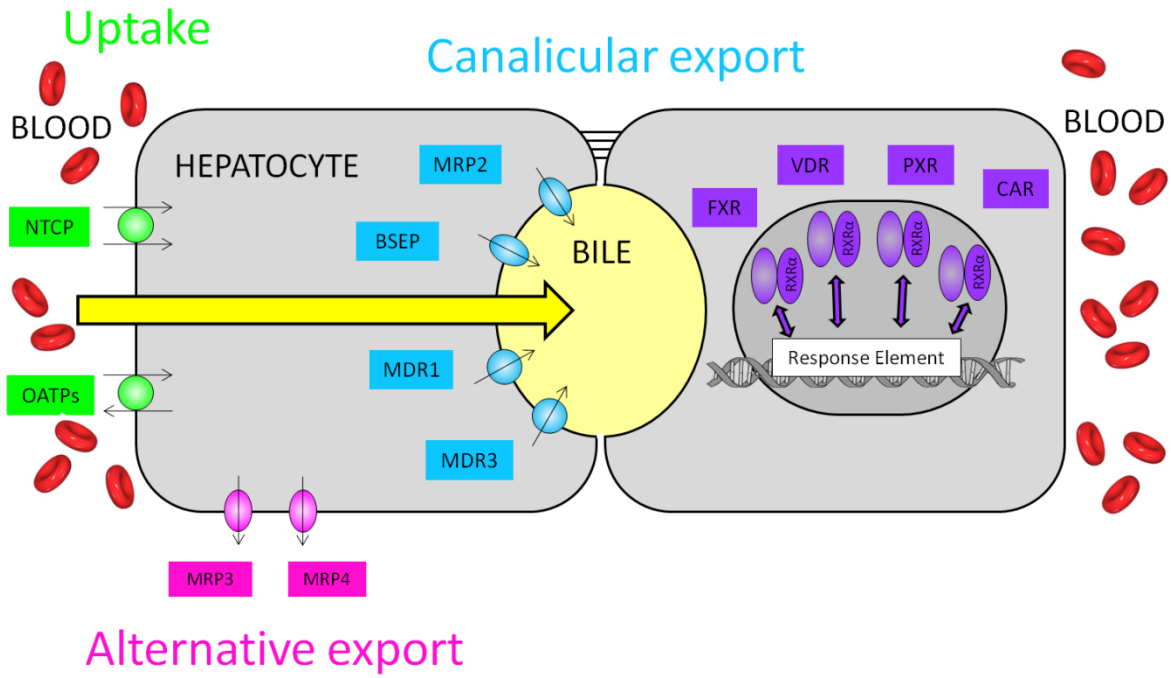


Figure 6.1 Normal hepato-billiary transport system: simplified scheme

Our study of serum analyses indicated that BA levels were highly elevated in critically ill patients on their last alive day in ICU. Surprisingly, while bilirubin levels increased 8-fold during critical illness, serum total BAs were 11-fold higher. Bilirubin was predominantly conjugated, and the larger increase in circulating total BAs mainly consisted of glycine and taurine conjugates of CA and CDCA, whereas unconjugated CA and CDCA did not differ from controls. This indicates that the hepatocytes were able to conjugate potentially toxic BAs, either de novo synthesized or entero-hepatically recirculated. It also suggests that the transport of the conjugated BA towards the apical bile canaliculi is strongly shifted to the blood (Figure 6.2).

Despite the strongly elevated serum BA levels during critical illness, CYP7A1, the rate limiting step in de novo BAs synthesis was only repressed at the mRNA level but not at the protein level. This is in line with the absence of increased SHP mRNA expression in ICU-patients, which mediates BA repression of CYP7A1 (8). Furthermore, FXR and its heterodimeric partner RXRα, which act in concert with SHP to suppress BA synthesis enzymes, were absent from the hepatocytic nucleus, where they exert transcriptional activity through direct binding to DNA. This may imply an at least partial loss of the sensing of BAs and its feedback regulation of de novo BA production, in light of the increased circulating BAs in ICU patients. Alternatively, critical illness may induce elevated BA levels by suppressing the BA sensor FXR and maintaining and/or shifting BA-synthesis.

BA and bilirubin are transported by the hepatocyte via the hepatobiliary transporters. The most prominent changes in the expression profile of the hepatic BA transporters during prolonged critical illness were observed in the basolateral efflux transporters MRP3 and MRP4 (Figure 6.2). Normally, MRP3 and MRP4 are expressed at very low levels in hepatocytes, but they become upregulated by inflammation and during longstanding cholestasis, presumably shifting transport of BAs back into sinusoidal blood for elimination by the kidneys (9). MRP3 correlated well with histological bilirubinostasis and serum bilirubin and conjugated BAs levels, suggesting that MRP3 upregulation is a compensatory reaction to cholestasis, as has been observed in animal bile duct ligation models of cholestasis (10). The upregulation of MRP3 (and MRP4) provides a mechanism to limit hepatocellular retention of hydrophobic BAs and other potentially toxic compounds that would normally be destined for biliary excretion.

Immunohistochemical expression of BSEP in the hepatocyte canalicular domain was dramatically reduced in ICU patients, especially in regions of bilirubinostasis. This decreased BSEP expression appears to be a major contributor to the cholestatic phenotype of the prolonged critically ill patient, as toxic BAs will accumulate within the hepatocytes.

In contrast to findings from chronic cholestatic disorders (9) and animal models of cholestasis (11) and sepsis (12), MRP2, the main canalicular bilirubin transporter, was upregulated during critical illness. This seems difficult to reconcile with the elevated serum bilirubin levels. Nevertheless, it may fit with the rather moderate increase in serum bilirubin, compared to the changes in serum BA concentrations. Besides, bile formation is a secretory process that depends on osmotically active solutes, mainly BAs. If the bile flow is hampered as a consequence of retained BAs, bilirubin will also be retained, essentially as a biochemical epiphenomenon. Canalicular MDR3 was also upregulated in

ICU patients. Given the key role of biliary phospholipids in protecting bile duct epithelium from the potentially toxic biliary content, upregulation of MDR3 might also exert a compensatory action, protecting the canalicular membrane and biliary epithelium.

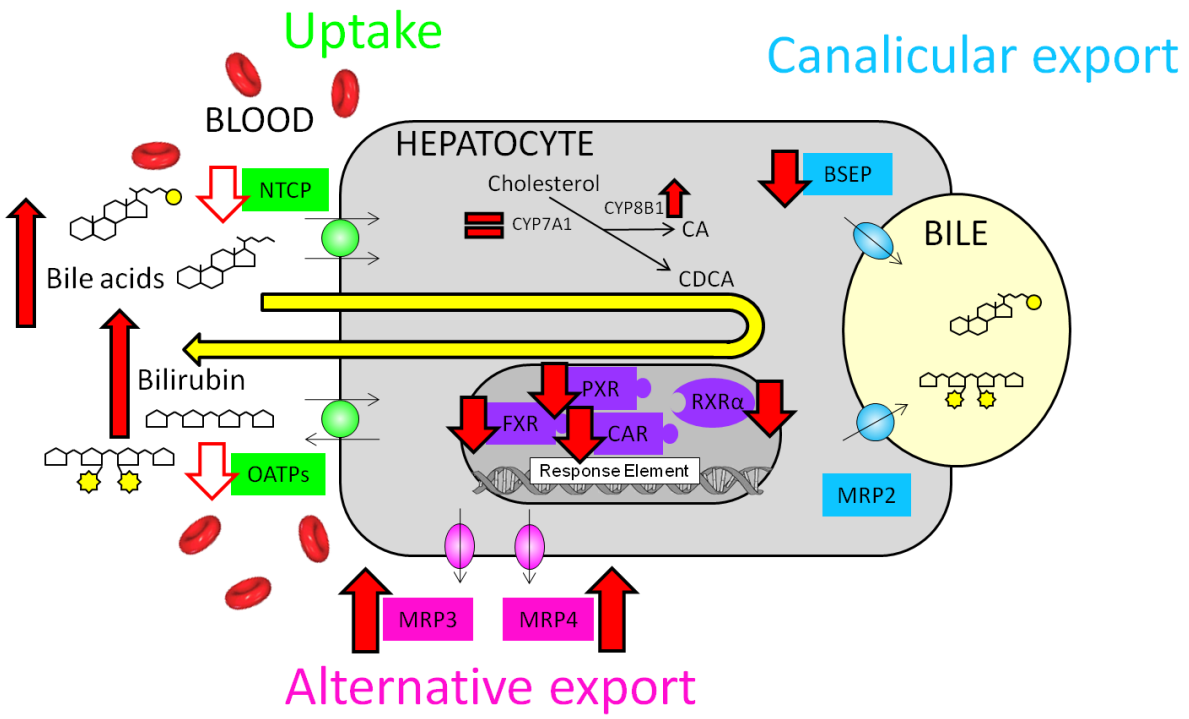


Figure 6.2 Observed alterations in the hepato-billiary transport system during critical illness

In summary, critical illness therefore is hallmarked by a strong increase in serum BA levels. Maintenance of BA-synthesis, suppression of FXR/RXRα, with lowering of apical BSEP and elevated basolateral MRP3 expression may either be a desired response during critical illness to raise serum BA concentrations or it may be a failing feed-back regulation on BA formation and disposition caused by cholestasis.

To further investigate whether the observed changes in the hepato-billiary transport system have to be interpreted as either adaptive or maladaptive, we evaluated cholestatic changes during critical illness in the context of a metabolic challenge (artificial nutrition versus nutritional restriction) known to influence the hepato-biliary transport system. Critical illness is often accompanied by anorexia and a failing of gastro-intestinal function. To prevent caloric deficits, when enteral nutrition is insufficient or poorly tolerated, administration of parenteral nutrition (PN) has been recommended, commencing as early as the first week of critical illness. PN is claimed to play a role in the

development of cholestatic liver dysfunction [13] and the mechanisms behind PN-induced cholestasis may include alterations in bile composition and transport as well as direct toxicity by bile acids to the hepatocytes. In the second (rabbit) study we therefore aimed to investigate whether fasting, by withholding PN, limits cholestatic liver dysfunction in a rabbit model of prolonged critical illness. We studied markers of hepatotoxicity, circulating bile acids and the hepatobiliary transport system. Critically ill rabbits were randomized to a nutritional strategy either accepting caloric deficits (Fasted, n=11) or covering caloric needs by PN (Fed, n=10).

Fasting during prolonged critical illness in rabbits resulted in decreased levels of AST and ALT, indicating suppressed parenchymal liver damage. Parenchymal liver damage during critical illness, also called hypoxic liver injury [14], is associated with poor outcome in the ICU [15].

In the critically ill rabbits we could not detect bilirubin in the serum, neither by the conventional enzymatic essays nor by High Performance Liquid Chromatography. For this reason we focused on the bile acids as markers of cholestasis. Withholding PN did not affect the concentration of total bile acids in the serum or in the liver. Nevertheless, fasting induced a shift from unconjugated CA and DCA to their glycine conjugated forms. This indicates a protective response as conjugated bile acids are less toxic than their unconjugated counterparts. It also corroborates our previous observation that in critically ill patients the unconjugated bile acids did not differ from controls, but went together with a large increase in the concentration of conjugated bile acids. Similarly as in study 1, the change in bile acid concentration could not be explained by increased de novo bile acid synthesis as the protein expression level of CYP7A1 was unaltered.
Fasting increased protein expression of the basolateral (MRP3) and the canalicular (BSEP) transporter, whereas the canalicular efflux pump MRP2 was suppressed. Gene expression levels of the nuclear receptor FXR were lower with fasting and correlated inversely with MRP3. The heterodimer partner of FXR, RXRA, was increased with fasting and correlated positively with MRP3. As we previously described discrepant responses between the gene and protein expression level, interpretation of the data should be done with caution [16]. However, taken that withholding PN during critical illness holds a beneficial response, reduced expression of the bile acid sensor FXR, maintenance of bile acid synthesis (CYP7A1) and reversal of bile acid transport (MRP3) may constitute a protective response.

The rabbit study clearly showed that reduced expression of nuclear bile acid sensors with maintenance of bile acid synthesis and reversal of bile acid transport to the blood compartment can be seen as a protective response, at least from the standpoint of the liver. However, whether the

increased circulating levels of bile acids and bilirubin, and the shift towards more conjugated bile acids in critically ill patients holds a survival benefit is not clear yet from this study.

We further investigated the relationship between biochemical cholestasis and nutrition in human patients. In a large randomized controlled multicenter trial we have recently assessed the outcome effect of tolerating a nutritional deficit during the first week in ICU (Late PN), compared with early initiation of PN to supplement insufficient enteral feeding (Early PN) [17,18]. Late PN enhanced organ function recovery, reduced the incidence of new infections and shortened duration of ICU stay. Strikingly, more patients in the late PN group had hyperbilirubinemia above 3 mg/dL, whereas fewer late PN patients had a clinically relevant increase in levels of GGT or ALP (19). These observations strengthen our postulated hypothesis and the observations made in study 2 that hyperbilirubinemia is brought about by an altered hepato-billiary transport system and may represent an adaptive response. Therefore, the aim of the third study of this thesis was to compare the effect of late versus early PN on circulating bilirubin, BAs and the liver enzymes GGT, ALP, ALT and AST over time. In addition, we evaluated the impact of late PN versus early PN on biliary sludge by ultrasonography on ICU day 5.

From day 1 after randomization until the end of the 7-day intervention window, bilirubin was higher in the late PN than in the early PN group. In the late PN group, as soon as PN was started on day 8, bilirubin fell and the two groups became comparable. In contrast, maximum levels of GGT, ALP and ALT were lower in the late PN group. Glycine/taurine-conjugated primary BAs increased over time in ICU, similarly for the two groups. Fewer patients in the late PN than in the early PN group developed biliary sludge on day 5.

Early PN during critical illness increased the levels of cholestatic liver enzymes GGT and ALP and increased the incidence of biliary sludge. The latter is in line with the previously observed association between long-term administration of PN and the development of biliary sludge in critically ill patients [20] and with the results from a rabbit experiment documenting gallbladder distension within 1 week of PN [21]. However, the association between PN and biliary sludge in the critical care setting has always been biased by the fact that more severely ill patients do not tolerate enteral feeding and rely on PN to maintain caloric intake. With the current study, a causal relationship was established. We demonstrated that postponing the administration of PN reduced the incidence of gallbladder sludge during critical illness. Biliary sludge thus appears to be in part a preventable complication of critical illness as metabolic interventions such as tolerating caloric deficits by late PN but also tight blood

glucose control [22] synergistically lowered the occurrence of gallbladder sludge. Whether the caloric restriction is bringing about this effect directly or indirectly through the decreased incidence of ICU acquired infections [23] and lowered antibiotic use [24] cannot be delineated from this study.
In contrast with the liver enzymes, peak total bilirubin levels, the archetypical biochemical marker of cholestasis during critical illness, remained lower in the patients exposed to the early administration of PN. Conversely, patients in the late PN group consistently revealed higher bilirubin levels coinciding with a better outcome (shorter ICU stay and less ICU-acquired infections). As soon as PN was started after 1 week in the late PN group, this difference was mitigated. It confirms our previous finding that 'cholestasis' defined by bilirubin levels > 3 x ULN is more frequent in the late PN group during the intervention window [25]. Hence, a rise in plasma bilirubin may be an adaptive response in the context of caloric restriction during critical illness. Also, in other population studies elevated bilirubin levels had a protective effect against coronary artery disease [26] and stroke [27]. Hyperbilirubinemia may exert its beneficial effect by improving endothelial function and decreasing oxidative stress [28]. Heme oxygenase-1, which is the rate-limiting enzyme in the bilirubin production, is protective in endothelial cells against toxicity associated with high glucose and oxidative stress [29]. Heme oxygenase knock-out mice have been demonstrated to have a higher mortality and more liver and kidney injury during endotoxic shock [30]. Additionally, bilirubin administration was shown to attenuate liver and kidney damage in animal models [31,32].

We thus clearly demonstrated that early initiation of parenteral nutrition in critically ill patients immediately suppressed plasma bilirubin concentrations without affecting the plasma concentrations of circulating bile acids. This lack of impact on circulating BAs may partially be explained by the selection of patients with a surgical contraindication to enteral feeding. Consequently, the enterohepatic BA cycle may have been impaired. Whether enteral nutrition would exert an additional effect on circulating BAs, cannot be addressed as the study was not randomized for enteral nutrition and the specific subset of patients for whom BAs were quantified, did not receive any enteral nutrition.

In conclusion, our findings indeed indicate that 'cholestasis' in the prolonged phase of critical illness is brought about by changes in the hepato-billiary transport system. Critical illness induces an increase in circulating conjugated bile acids and bilirubin, apparently brought about by a reversal of normal bile acid transport to the blood. The hepatocyte appears to switch off the nuclear bile acid sensors in order to increase circulating bile acids. This energy-saving strategy of the hepatocyte may fit well in the concept of MODS as an adaptive, hibernating state. Instead of trying to transport the

conjugated bile acids, bilirubin and partially detoxified toxins in the bile against a steep concentration gradient at the expense of high ATP-use, it may be better for the liver and the body to accept higher circulating concentrations of bile acids, bilirubin and the endo/exotoxins. A simple description of the changes in the hepato-biliary transporters and their regulatory network will not improve patient outcome. Avoiding the need for the 'adaptive' response in the liver may be possible by modifying risk factors for development of ICU cholestasis, such as parenteral nutrition.

The observation that lowering or delaying the administration of parenteral nutrition during critical illness lowered the peak levels of liver enzymes AST and ALT in rabbits and GGT and ALP in human patients shows that nutrient restriction lowers mild hepatocyte lysis and/or cholestasis. Furthermore, nutrient restriction also reduced the incidence of biliary sludge. In contrast, bilirubin in human patients and conjugated bile acids in rabbits were further increased by nutrient restriction. Reversal of the hepato-billiary transport system seems to drive these biochemical changes during fasting. These observations strengthen our hypothesis that early mild hyperbilirubinemia may be a protective response of the liver and does not necessarily reflect cholestasis. Nevertheless, only an interventional study, in which circulating levels of bilirubin are actively manipulated, can answer whether mild hyperbilirubinemia truly improves patient-centered outcome during critical illness.

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Summary

Up to 20% of the patients in the intensive care unit develop cholestatic liver dysfunction (CLD), which has been associated with an increased risk of mortality. Cholestasis can be defined as a decrease in bile flow due to either obstruction of bile flow through mechanical obstruction of the hepatic bile ducts or to an inability of the hepatocyte to secrete bile into the bile duct. Mechanical obstruction of the extrahepatic bile duct is easily and robustly diagnosed by ultrasonography, but is only a rare cause of CLD in critically ill patients. In contrast, critical illness related CLD is predominantly due to intrahepatic non-obstructive cholestasis, leading to an intracellular accumulation of bilirubin and bile acids. Although increased concentrations of bile acids inside the hepatocytes are presumed to induce cholestatic liver damage, the molecular and biochemical changes underlying CLD are poorly characterized and clear diagnostic criteria are lacking. The most widely used definition of CLD in clinical practice is a hyperbilirubinemia above 3 mg/dL. However, as a causal link between hyperbilirubinemia and worse outcome is missing, it may even be a biochemical epiphenomenon. Furthermore, hyperbilirubinemia might represent an adaptive response, as bilirubin can act as an endogenous anti-oxidant and may counteract the pro-oxidative effects of BA. Additionally, the reliability of hyperbilirubinemia as a marker of cholestasis in critically ill patients may be questionable, since there are many factors that can influence the levels of bilirubin.

In this thesis, we therefore aimed at further understanding the cholestatic changes at biochemical and molecular level during critical illness. The central hypothesis of this doctoral research project states that ‘cholestasis’, defined as ‘hyperbilirubinemia’ in the early phase of critical illness is brought about by changes in bile acid synthesis and transport and is a protective response of the liver. As parenteral nutrition (PN) is assumed to aggravate both CLD and biliary sludge formation, we further postulate that parenteral nutrition could modify this protective cholestatic response.

In our first study we examined a large cohort of critically ill patients of whom liver biopsies were available to gain mechanistic insights into ICU jaundice, with a focus on bile acids (BAs), hepatocytic transporters involved in bile production as well as their regulating nuclear receptors. Critically ill patients displayed elevated BA (11-fold) and bilirubin (8-fold) serum levels on their last alive day in ICU. Predominantly the conjugated fraction of both bilirubin and bile acids was elevated. This indicates that the hepatocytes were able to conjugate potentially toxic BAs, either de novo synthesized or entero-hepatically recirculated. Despite the strongly elevated serum BA levels during critical illness, CYP7A1, the rate limiting step in de novo BAs synthesis, was not increased. The reduced hepatocytic nuclear presence of FXR and RXRα, and a decreased expression of SHP, may imply at least a partial loss of the sensing of BAs and its feedback regulation of de novo BA production during critical illness. Alternatively, critical illness may induce elevated BA levels by

suppressing the BA sensor FXR and maintaining and/or shifting BA-synthesis. Immunohistochemical expression of BSEP in the hepatocyte canalicular domain was dramatically reduced in ICU-patients, especially in regions of bilirubinostasis. This decreased BSEP expression appears to be a major contributor to the cholestatic phenotype of the prolonged critically ill patient, as toxic BAs will accumulate within the hepatocytes. The most prominent changes in the expression profile of the hepatic BA transporters during prolonged critical illness were however observed in the basolateral efflux transporters MRP3 and MRP4. Normally, MRP3 and MRP4 are expressed at very low levels in hepatocytes, but they become upregulated by inflammation and during longstanding cholestasis, presumably shifting transport of BAs back into sinusoidal blood for elimination by the kidneys. MRP3 correlated well with histological bilirubinostasis and serum bilirubin and conjugated BAs levels, suggesting that MRP3 upregulation is a compensatory reaction to cholestasis. The upregulation of MRP3 (and MRP4) provides a mechanism to limit hepatocellular retention of hydrophobic BAs and other potentially toxic compounds that would normally be destined for biliary excretion.

To further investigate whether the observed changes in the hepato-billiary transport system have to be interpreted as either adaptive or maladaptive, we evaluated cholestatic changes during critical illness in the context of a metabolic challenge (artificial nutrition versus nutritional restriction) known to influence the hepato-biliary transport system. Critical illness is often accompanied by anorexia and a failing of gastro-intestinal function. To prevent caloric deficits, when enteral nutrition is insufficient or poorly tolerated, administration of parenteral nutrition (PN) has been recommended, commencing as early as the first week of critical illness. PN is claimed to play a role in the development of cholestatic liver dysfunction and the mechanisms behind PN-induced cholestasis may include alterations in bile composition and transport as well as direct toxicity by bile acids to the hepatocytes. In the second study of this thesis, we aimed to investigate whether fasting, by withholding PN, limits cholestatic liver dysfunction in a rabbit model of prolonged critical illness. We studied markers of hepatotoxicity, circulating bile acids and the hepatobiliary transport system. Critically ill rabbits were randomized to a nutritional strategy either accepting caloric deficits or covering caloric needs by PN. Fasting during prolonged critical illness in rabbits resulted in decreased levels of AST and ALT, indicating suppressed parenchymal liver damage. Fasting also induced a shift from unconjugated to conjugated BA. Similarly as in study 1, the change in bile acid concentration could not be explained by increased de novo bile acid synthesis. In contrast, fasting increased protein expression of the basolateral MRP3 and the canalicular BSEP transporter, whereas the canalicular efflux pump MRP2 was suppressed. Gene expression levels of the nuclear receptor FXR were lower with fasting and correlated inversely with MRP3. The heterodimer partner of FXR, RXRA, was increased with fasting and correlated positively with MRP3. As we previously described discrepant

responses between the gene and protein expression level, interpretation of the data should be done with caution. However, taken that withholding PN during critical illness holds a beneficial response on parenchymal liver damage, the observed changes may constitute a protective response, at least from the standpoint of the liver. However, whether the increased circulating levels of bile acids and bilirubin, and the shift towards more conjugated bile acids in critically ill patients holds a survival benefit is not clear yet from this study.

In a large randomized controlled multicentre trial we have recently assessed the outcome effect of tolerating a nutritional deficit during the first week in ICU (Late PN), compared with early initiation of PN to supplement insufficient enteral feeding (Early PN). Late PN enhanced organ function recovery, reduced the incidence of new infections and shortened duration of ICU stay. Strikingly, more patients in the late PN group had hyperbilirubinemia above 3 mg/dL, whereas fewer late PN patients had a clinically relevant increase in levels of GGT or ALP. These observations strengthen our postulated hypothesis that hyperbilirubinemia may represent an adaptive response. Therefore, the aim of the present study was to compare the effect of late versus early PN on circulating bilirubin, BAs and the liver enzymes GGT, ALP, ALT and AST over time. In addition, we evaluated the impact of late PN versus early PN on biliary sludge by ultrasonography on ICU day 5. Early PN during critical illness increased the levels of cholestatic liver enzymes GGT and ALP and increased the incidence of biliary sludge. In contrast with the liver enzymes, peak total bilirubin levels, the archetypical biochemical marker of cholestasis during critical illness, remained lower in the patients exposed to the early administration of PN. Conversely, patients in the late PN group consistently revealed higher bilirubin levels coinciding with a better outcome (shorter ICU stay and less ICU-acquired infections). Hence, a rise in plasma bilirubin may be an adaptive response in the context of caloric restriction during critical illness. Also in other population studies elevated bilirubin levels had a protective effect against coronary artery disease and stroke. Hyperbilirubinemia may exert its beneficial effect by improving endothelial function and decreasing oxidative stress.

In conclusion, our findings indeed indicate that ‘cholestasis’ in the early phase of critical illness is brought about by changes in the hepato-billiary transport system. Critical illness induces an immediate increase in circulating conjugated bile acids and bilirubin, apparently brought about by a reversal of normal bile acid transport to the blood. The hepatocyte appears to switch off the nuclear bile acid sensors in order to increase circulating bile acids. Avoiding the need for the ‘adaptive’ response in the liver may be possible by modifying risk factors for development of ICU cholestasis, such as parenteral nutrition. The observation that lowering or delaying the administration of parenteral nutrition during critical illness lowered the peak levels of liver enzymes AST and ALT in

rabbits and GGT and ALP in human patients shows that nutrient restriction lowers mild hepatocyte lysis and/or cholestasis. Furthermore, nutrient restriction also reduced the incidence of biliary sludge. In contrast, bilirubin in human patients and conjugated bile acids in rabbits were further increased by nutrient restriction. Reversal of the hepato-billiary transport system seems to drive these biochemical changes during fasting. These observations strengthen our hypothesis that early mild hyperbilirubinemia may be a protective response of the liver and does not necessarily reflect cholestasis. Nevertheless, only an interventional study, in which circulating levels of bilirubin are actively manipulated, can answer whether mild hyperbilirubinemia truly improves patient-centered outcome during critical illness.

Samenvatting

Van patiënten opgenomen op de intensieve zorgenafdeling zal ongeveer 20% cholestatische leverdisfunctie (CLD) ontwikkelen, een conditie die sterk geassocieerd is met een verhoogde mortaliteit en verlenging van de verblijfsduur. Cholestase wordt gedefinieerd als een verminderde galdoorstroming die ofwel veroorzaakt wordt door een mechanische obstructie van de galkanalen ofwel doordat de hepatocyt niet langer in staat is om gal te secreteren in de galkanalen. Mechanische obstructie is eenvoudig en betrouwbaar te diagnosticeren met behulp van echografie, maar is slechts zelden de oorzaak van CLD in kritiek zieke patiënten. CLD gerelateerd aan kritieke ziekte is daarentegen voornamelijk te wijten aan intrahepatische non-obstructieve cholestase, die leidt tot de accumulering van bilirubine en galzouten in de hepatocyt. Hoewel verondersteld wordt dat een verhoogde concentratie aan galzouten in de hepatocyt cholestatische leverschade veroorzaakt, is er zeer weinig geweten over de moleculaire en biochemische veranderingen die aan CLD voorafgaan. Bovendien ontbreken duidelijke diagnostische criteria. De meest courant gebruikte definitie om CLD in te klinische praktijk te definiëren, is een hyperbilirubinemie boven 3 mg/dL. Nochtans kon een causaal verband tussen hyperbilirubinemie en toegenomen sterfte nog niet worden aangetoond. Hierdoor kan niet uitgesloten worden dat hyperbilirubinemie slechts een biochemisch epifenomeen is. Daarenboven zou hyperbilirubinemie ook een adaptieve respons kunnen zijn, doordat bilirubine o.a. kan optreden als een endogene anti-oxidant en pro-oxidatieve effecten van galzouten kan tegenwerken. Bovendien is de betrouwbaarheid van hyperbilirubinemie als klinische merker voor cholestase in kritiek zieke patiënten bediscussieerbaar, aangezien er zeer veel factoren zijn die de bilirubine-serumspiegels kunnen beïnvloeden.

Het doel van deze thesis was daarom om cholestatische veranderingen op biochemisch en moleculair vlak tijdens kritieke ziekte in kaart te brengen. De centrale hypothese van dit doctoraat stelt dat ‘cholestase’, gedefinieerd als ‘hyperbilirubinemie’ in de vroege fase van kritieke ziekte veroorzaakt wordt door veranderingen in galzuursynthese en -transport en een beschermende respons is van de lever. Omdat de toediening van parenterale voeding tijdens kritieke ziekte verondersteld wordt om zowel CLD als de vorming van galblaassludge te verergeren, postuleren we bovendien dat de toediening van parenterale voeding deze protectieve cholestatische respons zal beïnvloeden.

In de eerste studie hebben we een grote groep kritiek zieke patiënten onderzocht van wie een post-mortem leverbiopsie beschikbaar was om zo mechanistisch inzicht te verwerven in ICU-gerelateerde CLD met een focus op galzouten, de hepatische transporters die betrokken zijn bij galproductie en de regulerende nucleaire receptoren. Kritiek zieke patiënten vertoonden verhoogde serumspiegels van galzuren (11-voudig) en bilirubine (8-voudig). Voornamelijk de geconjugeerde fractie van zowel bilirubine als de galzouten was verhoogd. Dit toont aan dat de hepatocyten nog in staat zijn om

potentieel toxische galzouten (ofwel 'de novo' aangemaakt ofwel entero-hepatisch gerecirculeerd) te conjugeren. Ondanks de sterk verhoogde galzoutspiegels was CYP7A1, het snelheidsbepalende enzyme in galzuursynthese, niet verhoogd tijdens kritieke ziekte. De verminderde nucleaire aanwezigheid van FXR en RXRA en de verminderde expressie van SHP impliceren mogelijks dat de hepatocyt een verstoorde werking vertoont van het feedback-systeem voor galzoutsynthese. Anderzijds zou het ook kunnen dat kritieke ziekte juist een verhoging van galzouten veroorzaakt door de 'galzout-sensor' FXR te verminderen en de galzoutsynthese in stand te houden. Immunohistochemische expressie van BSEP langs de canaliculaire zijde van de hepatocyt was sterk verminderd bij kritiek zieke patiënten, vooral in gebieden met aantoonbare bilirubinostasis. Deze verminderde BSEP-expressie lijkt dan ook een belangrijke rol te spelen in de ontwikkeling van het cholestatisch fenotype van de verlengd kritiek zieke patiënt, aangezien dit leidt tot de accumulering van toxische galzouten in de hepatocyt. De meest uitgesproken verandering in het expressiepatroon van de hepatische galzouttransporters was echter zichtbaar bij de basolaterale efflux-transporters MRP3 and MRP4. Normaal worden MRP3 and MRP4 slechts in zeer lage mate geëxprimeerd, maar de expressie stijgt tijdens inflammatie en langdurige cholestase, wat kan zorgen voor een ommekeer van het galzout-transport naar het bloed om dan via de nieren geëlimineerd te kunnen worden. In kritiek zieke patiënten correleerde MRP3 sterk met histologische bilirubinostasis, serum bilirubine en geconjugeerde galzuren, wat suggereert dat MRP3-opregulering een compensatoire reactie is op cholestase. De opregulering van MRP3 (en MRP4) is inderdaad potentieel een beschermend mechanisme om hepatocellulaire opstapeling van hydrofobe galzouten en andere toxische producten tegen te gaan die normaal via de biliaire weg afgevoerd worden.

Om verder te onderzoeken of de geobserveerde veranderingen in het hepato-biliair transportsysteem geïnterpreteerd moeten worden als adaptief dan wel maladaptief, hebben we cholestatische veranderingen tijdens kritieke ziekte verder onderzocht in de context van een metabole uitdaging (artificiële voeding versus nutritionele restrictie) waarvan geweten is dat deze het hepato-biliair transportsysteem beïnvloedt. Kritieke ziekte gaat vaak samen met anorexia en een falende gastro-intestinale functie. Om een calorisch deficit te vermijden, wanneer enterale voeding onvoldoende is of niet wordt getolereerd door de patiënt, wordt vaak overgestapt op het gebruik van parenterale voeding. Parenterale voeding zou echter een rol kunnen spelen in de ontwikkeling van CLD, het kan een invloed uitoefenen op de samenstelling van gal en op galtransport, alsook meer direct op de toxiciteit van galzouten in de hepatocyt. Daarom onderzochten we in de tweede studie van deze thesis of 'vasten', door geen (par)enterale voeding toe te dienen, de ontwikkeling van CLD zou verminderen. We maakten voor deze studie gebruik van een konijnen-model van verlengd kritieke ziekte om merkers van hepatotoxiciteit, circulerende galzouten en het hepatobiliair transportsysteem te bestuderen. Kritiek zieke konijnen werden gerandomiseerd voor een

voedingsschema waarbij ofwel een calorisch deficit werd getolereerd ofwel PN werd toegediend om tegemoet te komen aan de dagelijkse normale nood aan calorieën. Vasten tijdens langdurig kritieke ziekte veroorzaakte een verlaging in AST en ALT wat aangeeft dat parenchymale leverschade werd tegen gegaan. Vasten leidde ook tot een omzetting van ongeconjugeerde tot geconjugeerde galzouten in het bloed. Net zoals in de eerste studie kon de veranderde concentratie van circulerende galzouten niet verklaard worden door een toegenomen *de novo* synthese. In tegendeel, vasten zorgde voor een verhoogde expressie van de basolaterale transporter MRP3 en de canaliculaire BSEP transporter en een verminderde expressie van de canaliculaire effluxpomp MRP2. Genexpressie van de nucleaire receptor FXR was verlaagd tijdens vasten en correleerde negatief met MRP3. De heterodimeer partner van FXR, RXRA, vertoonde verhoogde expressie tijdens vasten en correleerde positief met MRP3. Bij de interpretatie van deze genexpressie-data moet echter rekening gehouden worden met eerder beschreven discrepante resultaten tussen genexpressie en eiwitexpressie. Desalniettemin, aangezien vasten tijdens kritieke ziekte een gunstig effect had op leverschade-parameters, kunnen de geobserveerde veranderingen inderdaad wijzen op een gunstige respons, tenminste vanuit het standpunt van de lever. Echter, of de stijging in galzouten en bilirubine, en de shift naar meer geconjugeerde galzouten in kritiek zieke patiënten een gunstige respons is, kan niet worden besloten uit deze studie.

In een grote gerandomiseerde studie, uitgevoerd door onze klinische onderzoeksgroep, werd recent het effect onderzocht van vroegtijdige toediening van parenterale voeding (vroege PN) versus het laattijdig (na 1 week in ICU) opstarten van parenterale voeding (late PN). Late PN versnelde het herstel en verminderde de kans op infecties. Verrassend was ook de bevinding dat meer patiënten in de late PN groep een hyperbilirubinemie boven 3 mg/dL vertoonden, terwijl minder patiënten in de late PN groep een klinisch relevante stijging vertoonden in GGT of ALP. Deze observaties versterken onze gepostuleerde hypothese dat hyperbilirubinemie een adaptieve respons zou zijn. Daarom was het doel van de laatste studie van deze thesis om het effect van late versus vroege PN na te gaan op het tijdsverloop van circulerend bilirubine, galzuren en de leverenzymes GGT, ALP, ALT en AST. We evalueerden ook het effect van late PN versus vroege PN op galblaassludge met behulp van echografie op de vijfde dag in ICU. Vroege PN tijdens kritieke ziekte verhoogde de serumspiegels van de cholestatische leverenzymes GGT en ALP en verhoogde de aanwezigheid van galblaassludge. In contrast met de leverenzymes bleef de piek-bilirubinewaarde, wat de meest gebruikte biochemische merker is voor cholestase tijdens kritieke ziekte, lager in patiënten in de vroege PN groep. Omgekeerd, patiënten in de late PN groep vertoonden hogere bilirubinespiegels gedurende de eerste week, en dit terwijl deze patiënten een beter herstel, een kortere ICU verblijfsduur en minder vaak nieuwe infecties vertoonden. Dit suggereert dat een stijging in plasma bilirubine een adaptieve respons is in de context van calorische restrictie tijdens kritieke ziekte. Ook in andere

patiëntenpopulaties werd eerder aangetoond dat verhoogde bilirubinespiegels een protectief effect hadden op de bloedvaten van het hart en de hersenen. Hyperbilirubinemie zou gunstig kunnen zijn door de endotheelfunctie te verbeteren en oxidatieve stress te verminderen.

In conclusie, onze bevindingen geven inderdaad aan dat 'cholestase' in de vroege fase van kritieke ziekte teweeg gebracht wordt door veranderingen in het hepato-biliair transportsysteem. Kritieke ziekte induceert een onmiddellijke stijging in circulerende geconjugeerde galzouten en bilirubine, een stijging die teweeg wordt gebracht door een ommekeer van het normaal galzout-transport naar het bloed. De levercel lijkt zijn nucleaire galzout-sensoren uit te schakelen om zo te zorgen voor verhoogde circulerende spiegels. Men zou de nood voor deze 'adaptieve' respons in de lever kunnen vermijden door te werken aan de risicofactoren om CLD te ontwikkelen, zoals bijvoorbeeld de toediening van parenterale voeding. De observatie dat het uitstellen of verlagen van de toediening van parenterale voeding tijdens kritieke ziekte leidde tot verlaagde piekwaarden van de leverenzymes AST en ALT in konijnen en GGT en ALP in humane patiënten, geeft aan dat voedingsrestrictie zorgt voor een verlaging van milde hepatocyt-lysis en/of cholestasis. Bovendien reduceerde voedingsrestrictie ook de ontwikkeling van galblaassludge. Nochtans, voedingsrestrictie verhoogde bilirubine in humane patiënten en geconjugeerde galzuren in konijnen. Omkeren van het hepato-biliair transport-systeem lijkt de oorzaak van deze veranderingen te zijn. Deze observaties ondersteunen dan ook onze hypothese dat een vroege milde hyperbilirubinemie een protectieve respons zou kunnen zijn van de lever en niet noodzakelijk cholestase aantoont. Alleen een interventionele studie echter, waarin de circulerende spiegels van bilirubine actief worden gemanipuleerd, zal een eenduidig antwoord kunnen geven of een milde hyperbilirubinemie het ziekteproces van de kritieke zieke patiënt gunstig kan beïnvloeden.

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Publications

Articles in internationally reviewed scientific journals

Vanwijngaerden YM., Wauters J, Langouche L, Vander Perre S, Liddle C, Coulter S, Vanderborght S, Roskams T, Wilmer A, Van den Berghe G, Mesotten D. Critical illness evokes elevated circulating bile acids related to altered hepatic transporter and nuclear receptor expression. Hepatology. 2011 Nov;54(5):1741-52

Mebis L., Eerdekens A., Guiza F., Princen L., Derde S., Vanwijngaerden YM., Vanhorebeek I., Darras V., Van den Berghe G, Langouche L . Contribution of nutritional deficit to the pathogenesis of the 'non-thyroidal illness syndrome' in critical illness: a rabbit model study. Endocrinology. 2012 Feb;153(2):973-84.

Boonen E,Vervenne H, Meersseman P, Andrew R, Mortier L, Declercq P, Vanwijngaerden YM, Spriet I, Wouters P, Vander Perre S, Langouche L, Vanhorebeek I, Walker B, Van den Berghe G. Mechanisms and Implications of Markedly Reduced Cortisol Metabolism during Critical Illness. N Engl J Med 2013 Apr 18;368(16):1477-88.

Vanwijngaerden YM, Langouche L, Derde S, Liddle C, Coulter S, Van den Berghe G, Mesotten D. Impact of parenteral nutrition versus fasting on hepatic bile acid production and transport in a rabbit model of prolonged critical illness. Shock 2013, Oct 1 [Epub ahead of print]

Vanwijngaerden YM, Langouche L, Brunner R, Debaveye Y, Gielen M, Casaer M, Liddle Ch, Coulter S, Wouters PJ, Wilmer A, Van den Berghe G, Mesotten D. Withholding parenteral nutrition during critical illness increases plasma bilirubin but lowers the incidence of biliary sludge. Hepatology 2013, Nov 9 [Epub ahead of print]

Book chapters, internationally recognised scientific publisher

Van den Berghe G., Vanwijngaerden YM., Mesotten D. ().Insulin therapy in the intensive care unit (2010). In: Wass A., Stewart P.(Eds), Oxford Textbook of Endocrinology and Diabetes, Chapt. 13.4.10.4 (pp. 1900-1905). Oxford University Press.

Gielen M., Vanwijngaerden YM., Mesotten D., Van den Berghe G. (2011). Hyperglycaemia and diabetes. In: Tubaro M., Danchin N., Filippatos G., Goldstein P., Vranckx P., Zahger D. (Eds.), European society of Cardiology Textbook of Intensive and Acute Cardiac Care, Chapt. 67 (pp. 698-708). Oxford University Press.

Meeting abstracts, presented at international conferences and symposia, published or not published in proceedings or journals

VanwijngaerdenYM., Wauters J., Mesotten D., Wouters P., Wilmer A., Van den Berghe G. (2008). Effects of tight glycemic control on cholestasis and biliary sludge in medical critically ill patients. Annual Conference on Shock by European Shock. Society Cologne, 28 June-2 July 2008. Poster presentation.

Vanwijngaerden YM., Wauters J., Langouche L., Vervenne H., Van den Berghe G., Mesotten D. (2009). Cholestasis of prolonged critical illness is largely explained by a strong upregulation of hepatic basolateral bile salts export pumps. The Endocrine Society's Annual Meeting. Washington, 10-13 June 2009. Poster presentation.

Vanwijngaerden YM., Derde S., Keereman A., Langouche L., Mebis L., Vervenne H., Van den Berghe G., Mesotten D. (2010). Impact of intravenous glucose load on bile salt transporters in critically ill rabbits. The International Liver Congress by the European Association for the Study of the Liver. Vienna, 14-18 April 2010. Poster presentation.

Vanwijngaerden YM, Wauters J, Langouche L, Vander Borght S, Roskams T, Liddle C, Coulter S, Wilmer A, Van den Berghe G, Mesotten D. (2010). A strong reduction in hepatic RXRα and CAR combined with an upregulation of MRP3 and MDR3 during prolonged critical illness may contribute to cholestasis. Benelux Nuclear Receptor Meeting by Nuclear Receptor Research Network, Ghent, 25 Oktober 2010. Poster presentation.

Boonen E, Vervenne H, Meersseman P, Mortier L, Vanwijngaerden YM, Spriet I, Langouche L, Vanhorebeek I, Van den Berghe G. (2012). Reduced cortisol metabolism drives hypercortisolism in critical illness. International Symposium on Intensive Care and Emergency Medicine. Brussels, 20-23 March 2012. Poster presentation.

Boonen E, Vervenne H, Meersseman P, Mortier L, Vanwijngaerden YM, Spriet I, Walker B, Langouche L, Vanhorebeek I, Van den Berghe G. (2012).Reduced cortisol metabolism as a driver of ACTH suppression during critical illness. Endocrine Society's Annual Meeting, Houston, 23-26 June 2012. Oral presentation.

Boonen E, Vervenne H, Meersseman P, Andrew R, Mortier L, Declercq P, Vanwijngaerden YM, Spriet I, Wouters P, Vander Perre S, Langouche L, Vanhorebeek I, Walker B, Van den Berghe G. (2012).Mechanisms and implications of markedly reduced cortisol metabolism during critical illness. Belgian Endocrine Society's Annual Meeting. Liege, 20 October 2012. Oral presentation.

Vanwijngaerden YM, Langouche L, Gielen M, Debaveye Y, Casaer M, Liddle C, Coulter S, Brunner R, Wouters P, Wilmer A, Van den Berghe G, Mesotten D. Withholding parenteral nutrition during the first week of critical illness increases plasma bilirubin but lowers the incidence of cholestasis and

gallbladder sludge. International Symposium on Intensive Care and Emergency Medicine. Brussels, 19-22 March 2013. Poster presentation.

Vanwijngaerden YM, Langouche L, Gielen M, Debaveye Y, Casaer M, Liddle C, Coulter S, Brunner R, Wouters P, Wilmer A, Van den Berghe G, Mesotten D. Withholding parenteral nutrition during the first week of critical illness increases plasma bilirubin but lowers the incidence of cholestasis and gallbladder sludge. 33rd Annual Meeting of the Belgian Society of Intensive Care Medicine, Brussels, 14 June 2013. Poster presentation.

Meeting abstracts, presented at local conferences and symposia, published or not published in proceedings or journals

Vanwijngaerden Y., Debuck F., Vandermeersch E., Van de Velde, M. (2007). Thrombocytopenia in patients with the diagnosis of preeclampsia: use of thromboelastography to guide the anaesthetic technique used. Research Meeting by UZ Gent, Ghent 16 June 2007. Oral presentation.